From: Dunbar, Anwar
To: Rowland, Grant
Subject: RE: DERs BCP
Date: Tuesday, April 21, 2015 10:51:00 AM
Attachments: 018986_2015-03-19_TXR0057111.pdf

Hey Grant. It looks like the records center compiled all of the ders into one chapter/file. See the attached pdf. There also appears to be a zip file in lotus notes as well were I suspect the individual word files are.

Anwar Y. Dunbar, Ph.D., Pharmacologist
Risk Assessment Branch 1
The Human Health Effects Division/ The Office of Pesticide Programs
1200 Pennsylvania Ave, NW
Washington, DC 20460

"Mastery of any cognitive skill requires roughly 10,000 hours of practice"- Malcolm Gladwell, Author of the book Outliers

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From: Rowland, Grant
Sent: Tuesday, April 21, 2015 10:44 AM
To: Dunbar, Anwar
Subject: DERs BCP

Anwar,
Scratch that last one. It looks like that is the ECO DERs. What was the date you uploaded the HED DERs?

-Grant

Grant Rowland
Herbicide Branch
Registration Division
Office of Pesticide Programs
703-347-0254

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

Date: 19-MAR-2015

SUBJECT: Bicyclopyrone; Review and generation of a Data Evaluation Records.

PC Code: 018986
Decision No.: NA
Petition No.: NA
Risk Assessment Type: NA
TXR No.: 0057111
MRID No.: See tables 1&2 below

DP Barcode: 425155
Registration No.: NA
Regulatory Action: NA
Case No.: NA
CAS No.: 352010-68-5
40 CFR: NA

FROM: Anwar Y Dunbar, Ph.D.
Pharmacologist, Risk Assessment Branch 1
Health Effects Division (HED) (7509P)

THROUGH: Charles W. Smith III, Branch Chief
Risk Assessment Branch 1/HED (7509P)

TO: Grant Rowland, Risk Manager Reviewer
Registration Division (7505P)

I. Conclusions

RAB1 has reviewed the DERs comprising the bicyclopyrone hazard database. The guideline studies used for risk assessment are all acceptable/guideline studies and satisfy the guideline requirements for each study type. The range-finding and mode of action studies are acceptable non-guideline.

II. Action Requested

Please review the DERs for the bicyclopyrone Hazard database.

Table 1. Subchronic, Chronic and Other Toxicity Profile.

Study Type	MRID No. (year)/ Classification
14-Day oral toxicity (Rat)	47841988 (2012) acceptable/non-guideline

Table 1. Subchronic, Chronic and Other Toxicity Profile.	
Study Type	MRID No. (year)/ Classification
28-Day oral toxicity (Dog)	47841973 (2012) acceptable/non-guideline
90-Day oral toxicity (mouse)	47842172 (2012) acceptable/guideline
90-Day oral toxicity (Rat)	47841975 (2012) Acceptable/guideline
90-Day oral toxicity (Dog)	47841976 (2012) acceptable/guideline
28-Day dermal toxicity (rat)	47841978 (2012) acceptable/guideline
Prenatal developmental (rats)	47841993 (2012) acceptable/guideline
Prenatal developmental (New Zealand rabbits)	47841996 (2012) acceptable/guideline
Prenatal developmental (Himalayan rabbits)	47841998 (2012) acceptable/guideline
Prenatal developmental (Himalayan rabbits)	47841999 (2012) acceptable/guideline
Reproduction and fertility effects (rat)	47842127 (2012) acceptable/guideline
Chronic toxicity (dog)	47841977 (2012) acceptable/guideline
Carcinogenicity (mouse)	47841987 (2012) acceptable/guideline
Chronic/ Carcinogenicity (rat)	47841985 (2012) acceptable/guideline
Bacterial Reverse Mutation Test	47841979 (2007) acceptable/guideline

Table 1. Subchronic, Chronic and Other Toxicity Profile.	
Study Type	MRID No. (year)/ Classification
Salmonella typhimurium and Escherichia coli Reverse Mutation Assay	47841980 (2010) acceptable/guideline
In Vitro Mammalian Cell Gene Mutation Test	47841981(1997) acceptable/guideline
Micronucleus Assay in Bone Marrow Cells of the Rat	47841984 (2008) acceptable/guideline
In Vivo Rat Liver Unscheduled DNA Synthesis Assay	47841983 (2007) acceptable/guideline
Acute neurotoxicity screening battery	4782002 (2012) acceptable/guideline
28-Day preliminary Subchronic neurotoxicity screening battery (rats)	47842140 (2012) acceptable/non-guideline
Subchronic neurotoxicity screening battery	4782004 (2012) acceptable/guideline
Metabolism and pharmacokinetics (rat)	47841961, 47841962, 47841963, 47841964, 47841965, 47842110 (2010) acceptable/guideline
Dermal penetration (rat)	47842239 (2009) acceptable/guideline
Immunotoxicity (Rats)	47842008 (2012) acceptable/guideline

Table 2. Mode Of Action Studies.	
Study Type	MRID No. (year) / Classification
Effect on Rat Thyroid Peroxidase Activity *in vitro*	47841989 (2012) acceptable/non-guideline
28-Day dietary thyroid mode of action study in rats	47841990 (2012) acceptable/non-guideline

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) Date: 03/19/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) Date: 3/19/15

TXR#: 0057111

DATA EVALUATION RECORD

STUDY TYPE: Subchronic Oral Toxicity in Rats; 870.3100; OECD 452

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Dose Range-finding (rat) (dietary administration) (no applicable guidelines)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Pinto P, 2007. NOA449280: 14 day preliminary dietary toxicity study in rats. Central Toxicology Laboratory, Macclesfield, UK. Laboratory Report No. KR1678-TEC, 12 September 2007. Unpublished. Syngenta File No. NOA449280/0060 MRID 47841988

SPONSOR: Syngenta Limited, Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK

COMPLIANCE: A signed and dated GLP statement was provided. The protocol, study procedures and report were not audited.

EXECUTIVE SUMMARY

In a 14-day subchronic oral feeding toxicity study (MRID #47841988), groups of four male, 5-6 week old HsdRccHan:WIST rats were fed diets containing 0 (control), 0.5 , 1, 2.5, 5 or 10 ppm bicyclopyrone (NOA449280, purity 94.5%) for 14 consecutive days. Actual test item intake/body weight/day was not calculated by the registrant.

Clinical observations, body weights and food consumption were measured throughout the study. At the end of the scheduled period, the animals were killed. Cardiac blood samples were taken for analysis of plasma tyrosine and bicyclopyrone. A necropsy was not conducted.

There were no deaths or clinical abnormalities during the study. There were no effects on bodyweight or food consumption.

A clear dose response was observed in both bicyclopyrone and tyrosine plasma concentrations. For a 20-fold increase in dose of bicyclopyrone from 0.5 ppm to 10 ppm, the achieved plasma concentration of bicyclopyrone increased 11.4-fold, and plasma tyrosine increased 5.7-fold.

The study was not conducted to assess the toxicological potential of bicyclopyrone, only its effects on plasma tyrosine levels. Thus no NOAEL and LOAEL are selected.

This study is classified as totally reliable (**acceptable/non-guideline)** and satisfies the guideline requirements (OPPTS 870.3100; OECD 408) for a subchronic oral range-finding toxicity study in rats.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Technical, brown beige powder
Lot/Batch number:	SEZ3AP006
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Expiry May 2007
Structure:	

Vehicle and/or positive control: The test substance was administered via RM1 diet (Special Diets Services Limited, Stepfield, Witham, Essex, UK).

Test Animals:

Species	Rat
Strain	HsdRCCHan : WIST
Age/weight at dosing	5-6 weeks/group mean range 87.3-89.3 g
Source	Harlan UK, UK
Housing	Group housed (up to 4 per cage) in metal cages
Acclimatisation period	At least 5 days
Diet	RM1 *ad libitum*
Water	Mains water *ad libitum*
Environmental conditions	Temperature: 22±3°C Humidity: 30-70% Air changes: At least 15 changes per hr Photoperiod: 12 hrs dark / 12 hrs light:

Study Design and Methods:

In-life dates: Start: 20 June 2006 25 July 2006

Animal assignment: The study comprised one replicate (randomised block), containing one cage per treatment group. Cages within the replicate were allocated to an experimental group based on a randomly generated Latin Square. The animals were allocated to the groups on the basis of pre experimental body weight.

Route and duration of administration: The test substance was administered in the diet to male rats (4/dose) for 14 days at 0, 0.5, 1.0, 2.5, 5 or 10 ppm.

Table 1: Study design

Test group	Dietary concentration (ppm)	Animal numbers
1	0 (control)	1-4

2	0.5	5-8
3	1	9-12
4	2.5	13-16
5	5	17-20
6	10	21-24

Table was taken from page 12 of the study report

Dose selection rationale: The dose levels were selected from previous toxicity studies with this test substance (CTL Study number KR1449, minimum dose level 50 ppm; CTL Study number PR1250 minimum dose level 500 ppm), in order to investigate the dose response of plasma tyrosine at low dietary inclusion levels.

Diet preparation and analysis: All experimental diets were based on RM1 diet, prepared prior to the start of the study and stored at nominally -20°C until required. Samples from all dietary levels (including control) were taken prior to the start of the study but were not analysed quantitatively for bicyclopyrone. During the study, diets were stored at room temperature.

Although no analysis of the diets were made, the test substance was shown to be present in the test diets as indicated by the presence of both bicyclopyrone and tyrosine in the plasma in a dose-related manner.

Observations: Detailed clinical observations, including the finding of 'no abnormalities detected', were recorded at the same time that the body weights were recorded.

Cage-side observations, which included recording any changes in clinical condition or behaviour, were made once daily.

Body weight: The body weight of each rat was recorded immediately before feeding of the experimental diets commenced and then on the same day of each subsequent week until termination.

Food consumption and test substance intake: Food consumption was measured weekly throughout the study by recording a given value at the beginning of the week and a residue at the end of the week. Where diet changes were required additional residue and given values were recorded during the week. The daily test substance intake was not reported.

Investigations *post mortem:* All rats were killed by over exposure to halothane Ph. Eur. vapour followed by exsanguination by cardiac puncture. Blood samples were taken for the assessment of plasma tyrosine. The animals were discarded following exsanguination.

Plasma tyrosine: Plasma tyrosine levels were assessed in the plasma from blood samples collected into tubes containing lithium heparin as an anticoagulant. Plasma concentrations of tyrosine were determined using a high performance liquid chromatography tandem MS-MS analytical method.

Plasma bicyclopyrone (NOA449280): Plasma bicyclopyrone levels were assessed in the plasma from blood samples collected into tubes containing lithium heparin as an anticoagulant. Plasma concentrations of bicyclopyrone were determined using a high performance liquid chromatography tandem MS-MS analytical method.

Statistics: Body weight data were evaluated using Students T-test.

RESULTS AND DISCUSSION

Mortality: None of the animals died.

Clinical observations: There were no clinical abnormalities recorded during the study

Body weight and weight gain: There were no differences from control for body weight or body weight gain during the study. Absolute body weights are presented in table 2.

Table 2: Intergroup comparison of absolute body weights (g)

	Dietary Concentration of bicyclopyrone (ppm)					
Day	**0**	**0.5**	**1**	**2.5**	**5**	**10**
1	88.9 ± 10.4	89.0 ± 4.3	88.3 ± 6.6	87.3 ± 6.3	88.6 ± 6.8	89.3 ± 6.0
8	124.0 ± 13.6	120.0 ± 9.6	122.3 ± 2.1	125.0 ± 5.3	125.3 ± 4.1	123.8 ± 8.5
15	157.5 ± 19.2	153.5 ± 11.4	148.5 ± 3.7	158.0 ± 8.1	161.3 ± 5.9	155.5 ± 11.1

Data were taken from page 18 of the study report. Standard deviations were not calculated by the registrant.

Food consumption and compound intake: There were no differences from control for food consumption during the study.

Bicyclopyrone in plasma: A clear dose response was observed when comparing nominal bicyclopyrone concentrations to actual bicyclopyrone plasma levels. For a 20-fold increase in dose from 0.5 ppm to 10 ppm the achieved plasma concentration of bicyclopyrone increased 11.4-fold (Table 3).

Plasma tyrosine: A clear dose response was also observed in tyrosine plasma concentrations when compared to the increases of the nominal concentrations bicyclopyrone. For a 20-fold increase in dose from 0.5 ppm to 10 ppm the achieved plasma concentration of plasma tyrosine increased 5.7-fold (Table 3).

Table 3: Plasma concentrations of bicyclopyrone (NOA449280) and tyrosine

	Dietary Concentration of bicyclopyrone (ppm)					
	0	**0.5**	**1**	**2.5**	**5**	**10**
Average NOA449280 (ng/mL)	<5	17.5	12.9	72.5	108	200
Average tyrosine (nmol/mL)	134	295	306	655	930	1697

Data were taken from pages 21-22, and 49-50 of the study report. Standard deviations were not calculated by the registrant.

INVESTIGATOR'S CONCLUSION: Dietary administration of up to 10 ppm bicyclopyrone for 14 consecutive days had no effect on body weight or food consumption.

There was a clear dose response with plasma concentrations of both bicyclopyrone and tyrosine increasing with dose. The tyrosine dose response profile mirrored the active ingredient profile well.

REVIEWER COMMENTS:

The study was not conducted to assess the toxicological potential of bicyclopyrone, only its effects on plasma tyrosine levels. Thus no NOAEL and LOAEL are selected.

This study is classified as totally reliable (**acceptable/non-guideline)** and satisfies the guideline requirements (OPPTS 870.3100; OECD 408) for a subchronic oral range-finding toxicity study in rats. EPA, PMRA (Canada), and APMVA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Pinto P, 2007)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar J. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) Date: 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) Date: 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

STUDY TYPE: Subchronic Oral Toxicity in Dog; 870.3150; OECD 452

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Subchronic Oral Toxicity; OPPTS 870.3150 [§82-1b].

TEST MATERIAL (PURITY): NOA449280 (96 % w/w)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Twomey K, 2003. NOA449280: 28 day oral toxicity study in dogs. Syngenta Limited, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK. Laboratory Report No. CTL/KD1487/TEC/REPT. 06 November 2003. Unpublished. (Syngenta File No. NOA449280/0018) MRID 47841973

COMPLIANCE: Signed and dated GLP statements were provided. No QA auditing was conducted for this study.

There were no deviations from similar regulatory guidelines considered to compromise the scientific validity of this study.

EXECUTIVE SUMMARY

In a 28-day subchronic oral toxicity study (MRID #47841973), 96% bicyclopyrone (NOA449280) was administered daily throughout the treatment period by oral capsule to groups of one male and one female beagle dogs, at 0 (control), 10, 100 or 250 mg/kg/day for a period of up to 4 weeks. Clinical observations and veterinary examinations (including ophthalmoscopy) were made and bodyweights, food consumption, clinical pathology and toxicokinetic parameters were measured periodically throughout the study. At the end of the scheduled period, the animals were killed and subjected to a full examination *post mortem*. Selected organs were weighed and specified tissues were taken for subsequent histopathology examination.

At 10 and 100 mg/kg/day bicyclopyrone, there were no treatment related effects.

The animals in the group administered bicyclopyrone at 250 mg/kg/day were killed ahead of schedule following adverse clinical signs, bodyweight loss and reduced food consumption for the

male on day 7 of the study. Various pathology parameters were affected including fluid effusion in the outer choroid of one eye, swelling and vacuolization of the retinal pigment epithelium, minimal inflammatory cell infiltration at the choroidal/retinal junction and partial retinal detachment. There were also reduced cortical lymphocytes in the thymus. Male number 7 also showed an increased level of plasma alkaline phosphatase (↑144%). Urine phenolic acid concentrations and plasma tyrosine and bicyclopyrone concentrations were also elevated. It was considered that this dose level exceeded the Maximum Clinically Tolerated Dose (MCTD).

As expected, based on the mode of action of this class of chemical, urine phenolic acid concentrations and plasma tyrosine and bicyclopyrone concentrations were elevated (data not shown in this DER).

Based upon the results of this study, the NOAEL is 100 mg/kg/day. The LOAEL is 250 mg/kg/day based upon clinical signs, bodyweight loss, reduced food consumption on day 7, and moderate retinopathy. Also in this 250 mg/kg/day male, various clinical pathology parameters were affected, and urine phenolic acid concentrations and plasma tyrosine and bicyclopyrone concentrations were elevated.

This study is classified as totally reliable (**acceptable/non-guideline**) and satisfies similar test guideline requirements (OPPTS 870.3150; OECD 409) for a subchronic oral range-finding toxicity study in the dog.

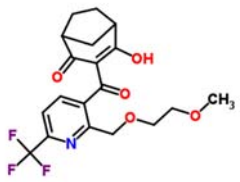
COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test material:	Bicyclopyrone (NOA449280)
Description:	off white
Lot/Batch number:	KI6353/13 (KI6353/19 from day 2)
Purity:	96%
Storage conditions:	Ambient temperature in the dark

Structure:



Vehicle: Gelatine capsule of approximately 5ml capacity (supplied by TORPAC Inc., East Hanover, New Jersey, USA).

Test Animals:	Dog
Strain:	Beagle

Age/Weight:	16-24 weeks at delivery, 9.1-10.7kg (males) and 6.8-8.4kg (females) at start of dosing
Source:	Conventional Animal Breeding Unit, Alderley Park, Macclesfield, Cheshire, UK
Housing:	L Block Unit 3, same sex pairs in indoor pens with sleeping platforms and interlinking gates.
Acclimatisation period:	At least 2 weeks
Diet:	Laboratory Diet supplied by Special Diet Services Limited, Stepfield, Witham, Essex, UK)
Water:	supplied by automatic system *ad libitum*
Environmental conditions:	Temperature: 19±4^{0}C Humidity:30-70% Air changes: approximately 15 hours Photoperiod: artificial giving 12 hours light, 12 hours dark.

Study Design and Method:

In life dates: Start: 01 May 2002 End: 22 July 2002

Animal assignment: In a subchronic toxicity study, dogs were randomly allocated to the treatment group and individually identified by tattooed ear numbers or implanted microchips.

The study consisted of four groups; one control and three receiving bicyclopyrone with one male and one female per group.

Route and duration of administration: The following groups were administered bicyclopyrone in in gelatin capsules for at least 4 weeks.

Table 1: Study design (animal assignment)

Group	Daily dose of Bicyclopyrone (mg/kg/day)	Experimental numbers	
		Males	Females
1	0	1	2
2	10	3	4
3	100	5	6
4	250	7	8

Data were taken from page 16 of the study report

Diet preparation and analysis: Dogs were dosed orally, immediately prior to feeding, in pen order at approximately the same time each day. Control animals were given empty gelatine capsules.

Observations: All dogs were observed at least three times daily for clinical behavioural abnormalities and, on a weekly basis, they were given a thorough examination. Gastrointestinal excreta were assessed daily, for at least 1 week pre-study and then throughout the treatment period. Assessments were made on an individual basis for up to 4 hours post-dosing. All dogs were also given a full clinical examination by a veterinarian pre-study and prior to termination. The examination included cardiac and pulmonary auscultation and indirect ophthalmoscopy.

Bodyweight: All dogs were weighed before feeding, on a weekly basis pre-study, on day 1 and thereafter at weekly intervals until termination.

Food consumption: Food residues were recorded daily, approximately 4 hours after feeding and any residual food was discarded. These measurements were made for at least 1 week pre-study and throughout the treatment period. Food consumption was calculated, at weekly intervals, as a mean value (g food/day) for each dog. Males consumed 300 g of food per day and females consumed 350 g.

Haematology: Jugular vein blood samples were taken before feeding from all dogs in week -1 and on days 8, 16, 22 and 29 (or prior to termination if not scheduled) into four tubes containing EDTA, trisodium citrate (x 2) or lithium heparin as anticoagulants. The following measurements were made or determined on samples collected using EDTA as an anticoagulant:

Haemoglobin	platelet count
Haematocrit	total white cell count
red blood cell count	differential white cell count
mean cell volume	blood cell morphology, including differential
mean cell haemoglobin	reticulocyte count
mean cell haemoglobin concentration	white cell count

Clotting measurements: Prothrombin time and activated partial thromboplastin time (with kaolin) were measured using samples of blood collected using trisodium citrate as an anti-coagulant. Blood films were prepared but not examined in this study.

Clinical chemistry: The following measurements were made on the plasma from blood samples collected in tubes containing lithium heparin as an anticoagulant:

urea	alkaline phosphatase activity
creatinine	aspartate aminotransferase activity
glucose	alanine aminotransferase activity
albumin	gamma-glutamyl transferase activity
total protein	calcium
cholesterol	phosphorus (as phosphate)
Triglycerides	sodium
total bilirubin	potassium
creatine kinase activity	chloride

Urine clinical chemistry: Individual urine samples were collected by catheterisation from all dogs in week -1, immediately pre-dosing on days 2, 9, 16, 23 and 28 (and/or immediately prior to termination). The following were measured/recorded:

colour (only if abnormal)	glucose
Volume	bilirubin

Appearance	protein
pH	ketones
specific gravity	blood
Creatinine	

Plasma toxicokinetic and tyrosine analysis: A jugular blood sample (approximately 1.3ml taken into tubes containing lithium heparin anti-coagulant) were taken from all dogs on day 1 at 0, 1, 2, 3, 4, 8, 12 and 24 hours post-dosing, immediately pre-dosing on days 4, 6, 8, 16, and on day 26 at 1, 2, 3, 4, 8, 12 and 24 hours post-dosing. The plasma was separated by centrifugation (1500g for 10 minutes) and the plasma (plus cells from the terminal samples) was then submitted for toxicokinetic and tyrosine analysis. Tyrosine was selected for analysis based on the mode of action of this class of chemical.

Urine analysis by NMR to determine urine levels of HPPA and HPLA: In the case of the phenolic acid metabolites (4-hydroxyphenylpyruvate [HPPA], 4- hydroxyphenyllactate [HPLA] and 4-hydroxyphenylacetate [HPAA]), the following formula was used to quantify them in the urine sample. This represents the total amount of free phenolic acids.

$$\text{conc phenolic acids(PA) mg/ml} = \frac{\text{Integral PA}/2}{\text{Integral TSP}/9} \times \frac{\text{Mol Wt PA}}{\text{Mol Wt TSP}} \times \text{conc TSP (mg/ml)}$$

$$(1) \quad = \frac{\text{Integral PA}}{\text{Integral TSP}} \times 4.73 \times \text{conc TSP (mg/ml)}$$

Assuming an "average" MW of 181 for the phenolic acids [reflects the major components, HPPA (180), HPLA (182)], the MW of TSP is 172.3.

For quantification in the aromatic region of the NMR spectra, the integral taken for HPPA, HPAA and HPLA was the two protons of the upfield half of the tyrosine ring centered at about 6.9 ppm.

When (HPLA) was quantified by reference to the proton attached to the alpha carbon (resonance at 4.25 ppm shift) the following formula was used:

$$\text{conc HPLA (mg/ml)} = \frac{\text{Integral HPLA (4.25 ppm)}}{\text{Integral TSP}} \times 9.46 \times \text{conc TSP(mg/ml)} \quad (2)$$

When (HPPA) was quantified in the aliphatic region, both methylene proton resonances were integrated hence formula (1) was used (integral at 4.02 ppm).

The results are finally expressed as a concentration (mmol/l) relative to the creatinine concentration (mmol/l) in the urine sample in order to normalize for the differing states of sample dilution.

Investigations *post mortem:* All dogs were killed by an overdose of anaesthetic induced by intravenous administration of sodium pentobarbitone followed by exsanguination.

Macroscopic examination: All animals were examined *post-mortem.* This involved an external observation and an internal macroscopic examination.

Organ weights: From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

adrenal glands	testes	liver (with gall bladder)
thyroid glands (with parathyroids)	Brain	ovaries
thymus	Heart	spleen
uterus (with cervix)	kidneys	

Tissue submission: The following tissues were examined *in situ*, removed and examined and fixed in an appropriate fixative:

abnormal tissues	oesophagus
adrenal gland (left)	ovary
aorta (abdominal)	oviduct
bone (femur and stifle joint)*	pancreas
bone and marrow (sternum)	parathyroid gland
bone marrow smear (rib)*	Peyer's patch
brain (cerebrum, cerebellum and medulla/pons)	pharynx*
Caecum	pituitary gland
Cervix	prostate gland
Colon	rectum
duodenum	salivary gland - submandibular
Epididymis	salivary gland - sublingual
eye (retina, optic nerve)	salivary gland - parotid
gall bladder	skin (lateral thigh)
Heart	spinal cord (cervical, thoracic, lumbar)
Ileum	spleen
Jejunum	stomach
Kidney	testis
larynx*	thymus
Liver	thyroid gland
Lung	trachea
lymph node (mesenteric)	urinary bladder
lymph node (cervical)	uterus
mammary gland (females only)	vagina
nasal passages*	voluntary muscle
nerve – sciatic	

* Tissues to be stored.

The tissues from the control group and the 100 and 250 mg/kg/day groups were submitted for histology and were routinely processed, embedded in paraffin wax, sectioned at 5µm and stained with haematoxylin and eosin.

Microscopic examination: All selected tissues from the control group, 100 and 250 mg/kg/day groups, together with all abnormalities were examined by light microscopy.

Data evaluation: Since there was a single animal per sex per group, data were evaluated by inspection and, if necessary, by comparison with historical control data.

RESULTS AND DISCUSSION

Mortality: Male number 7 (250 mg/kg/day) was killed *in extremis*, five and three quarters hours post-dosing on day 7 due to the clinical signs observed, described below.

Clinical observations: Male number 7 (250 mg/kg/day) was observed on day 7 with the following clinical signs: moderate hypoactivity, slightly unsteady gait and was unstable one hour post-dosing. Approximately three and half hours post-dosing the same clinical signs were displayed as well as increased heart rate and regurgitation and the animal was also vomiting four and three quarter hours post-dose. Five and a quarter hours post-dosing, the animal was displaying the same clinical signs and slight salivation.

The female in this dose group (250 mg/kg/day) did not show any clinical signs, it was considered that this dose level would not be used and had fulfilled its purpose for this study; therefore the female was killed for humane reasons during the second week of treatment.

Male number 5 (100 mg/kg/day) was observed with dry sores and scabs (head and thorax) and reddened ears periodically during the study. This was diagnosed as otitis and dermatitis and was considered not to be attributable to test substance administration.

Female number 6 (100 mg/kg/day) was also observed with reddened ears (otitis), during the first two weeks of treatment. This female was observed as thin in clinical observations during weeks 4 and 5.

There were no further clinical signs observed that were considered to be attributable to test substance administration.

Veterinary examination and ophthalmoscopy: There were no abnormal findings at veterinary examination or during ophthalmoscopy for the control group animals and those in the 100 mg/kg/day. There were no treatment related findings in the 250 mg/kg/day female.

The 250 mg/kg/day male was observed on day 7 of the study approximately one and a quarter hours post dosing with the pupillary reflex present but with both pupils failing to dilate following Mydracil application. Retinal details were hard to distinguish due to the cloudy appearance

behind the iris. The dog was very subdued, unsteady, had not eaten and had its abdomen tucked up (this was not painful on palpation). The heart rate was rapid, pulse strong and the heart sound were loud and audible all over the chest area. Approximately five and a half hours post dosing, the optic disc and vessels were visible, although hard to examine due to nystagmus. The dog was observed with a swaying gait, splayed hindlimbs, the head was low and swaying from side to side, slight opisthotonous and nystagmus, with salivation and ataxia and was therefore killed due to the clinical signs observed.

Gastro-intestinal findings: Male number 5 (100 mg/kg/day) was observed with fluid faeces and male number 7 (250 mg/kg/day) was observed to regurgitate on day 7. There were no further gastrointestinal findings.

Body weight and organ weights: There was no effect of bicyclopyrone administration on bodyweight at dose levels up to 100mg/kg/day. Male number 7 (250mg/kg/day) had lost approximately 0.8 kg bw from the previous week prior to termination. See table 2.

Spleen weight for the male and the female in the 250 mg/kg/day group were markedly lower than those of the control group. All other organ weights were similar to those of the control group and/or were considered to represent natural variation.

Table 2: comparison of bodyweights (kg)

	Bicyclopyrone (mg/kg/day)							
	Males				Females			
Week Number	0	10	100	250[b]	0	10	100	250[b]
-1	10.70	8.90	8.70	10.50	7.90	8.10	6.70	7.30
1	10.70	9.30	9.10	10.60	7.90	8.40	6.80	7.60
2	10.60	9.40	9.10	9.80	8.00	8.50	7.00	7.70
3	11.10	9.60	9.30	NA	8.20	8.60	7.10	NA
4	11.30	10.10	9.60	NA	8.40	8.80	7.20	NA
5	11.70	10.00	9.80	NA	8.60	9.00	7.30	NA

Data were taken from page 46 of the study report
a- Only one animal was tested per dose.
b- The animals in the group administered NOA bicyclopyrone at 250 mg/kg/day were killed ahead of schedule following adverse clinical signs, bodyweight loss and reduced food consumption for the male on day 7 of the study.

Food consumption: There was no effect of bicyclopyrone administration on food consumption at dose levels up to 100mg/kg/day. The food consumption for male number 7 (250mg/kg/day) was reduced on day 7.

Haematology: The only haematological abnormalities (increased haemoglobin, red blood cell count and haematocrit) noted were in male number 7 (250 mg/kg/day) just prior to it being killed for humane reasons on day 7 but were indicative of dehydration and possible as a result of stress. There were no changes of note for the female in this group. There were no obvious effects of any magnitude which were consistent between the sexes on the haematological parameters measured following bicyclopyrone administration at dose levels of up to 100 mg/kg/day for 28 consecutive days.

Blood clinical chemistry: Immediately prior to being killed for humane reasons on day 7, male 7 (250 mg/kg/day) showed increased alkaline phosphatase (ALP, ↑144%), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity and abnormalities in electrolyte levels (sodium, potassium and chloride reduced), all of which were associated with the dog's terminal condition. There were no changes in liver weight or pathology to support that the changes in enzymes is likely due to the state of the animal rather than being treatment related. Finally, plasma tyrosine levels were elevated in all treatment groups (data not shown). See table 3.

Table 3: Mean of selected clinical chemistry parameters

	Bicyclopyrone (mg/kg/day)							
	Males				**Females**			
Parameter	**0**	**10**	**100**	**250**	**0**	**10**	**100**	**250**
Total protein	53.2	54.5	57.22	64.1	54.12	55.96	51.54	54.97
Albumin	30.88	30.72	32.92	34.9	31.98	31.98	32.84	33.43
AST	26.4	35.4	28.4	64.5 (↑144%)	32	30.2	34.6	34
Glucose	6.3	6.04	6.26	7.25	6.1	5.78	5.86	7.23

Data were taken from pages 62-65 of the study report

Urine clinical chemistry: There were no apparent effects of bicyclopyrone administration on urine clinical chemistry parameters at dose levels of up to 100 mg/kg/day for 28 days and at 250 mg/kg/day for up to one week.

Urine analysis by NMR to determine urine levels of HPPA and HPLA: Phenolic acid metabolites (4-hydroxyphenylpyruvate [HPPA], 4- hydroxyphenyllactate [HPLA] and 4-hydroxyphenylacetate [HPAA]) were present in the urine of all of groups following dosing with bicyclopyrone. The control phenolic acid concentration was low on days –2, 2, and 9 of the study, however a rise was observed in male urine on days 16 and 23 and for female urine on days 16, 23 and 28 of the study at all doses tested (data not shown).

Macroscopic findings: There are no changes observed at scheduled examination of *post mortem* that were considered to be related to bicyclopyrone administration.

Microscopic findings: In the male dog dosed at 250 mg/kg/day, there was a moderate retinopathy characterised by fluid effusion in the outer choroid of one eye, swelling and vacuolation of the retinal pigment epithelium, minimal inflammatory cell infiltration at the choroidal/retinal junction and partial retinal detachment. In addition there were reduced cortical lymphocytes in the thymus. It is considered that these changes were related to test substance administration.

The finding of alveolitis in the lung of this dog in relation to test substance administration is doubtful as it is not uncommon to find this change as a spontaneous event.

No change was observed in the brain.

Although a variety of pathological findings was observed in other dogs, none were considered to be test substance-related as they were findings commonly seen in this age and strain of animal or were findings without a clear dose relationship.

INVESTIGATOR'S CONCLUSIONS: Repeat oral administration of 250 mg/kg/day bicyclopyrone to dogs exceeded the MCTD and this group was terminated ahead of schedule.

The highest dose level recommended for any subsequent repeat dose toxicity study in the dog should be less than 250 mg/kg/day.

REVIEWER COMMENTS:

Based upon the results of this study, the NOAEL is 100 mg/kg/day. The LOAEL is 250 mg/kg/day based upon clinical signs, bodyweight loss and reduced food consumption on day 7, and moderate retinopathy. Also in this 250 mg/kg/day male, various clinical pathology parameters were affected, and urine phenolic acid concentrations and plasma tyrosine and bicyclopyrone concentrations were elevated.

This study is classified as totally reliable (**acceptable/non-guideline**) and satisfies similar test guideline requirements (OPPTS 870.3150; OECD 409) for a subchronic oral range-finding toxicity study in the dog. EPA, PMRA (Canada), and APMVA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Twomey K, 2003)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar J. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

STUDY TYPE: 90-Day Mouse Preliminary Carcinogenicity Study OECD 408 (1998): OPPTS 870.3100 (1998): JMAFF 12 Nousan No 8147 (2000)

PC CODE: 018986 **DP BARCODE:** D425155

TEST MATERIAL (PURITY): NOA449280 (94.5%)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Shearer J & Wood M, 2009. NOA449280 - 90 Day Mouse Preliminary Carcinogenicity Study. Charles River, Tranent, Edinburgh, EH33 2NE, UK. Laboratory Report No. 28445. 22/07/2009. Unpublished. (Syngenta File No. NOA449280_11014) MRID 47842172

SPONSOR: Syngenta Limited, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study, although it should be noted that standard clinical chemistry and urinalysis parameters were not analysed in this study.

EXECUTIVE SUMMARY

In a subchronic oral toxicity study in mice (MRID #47842172), groups of 10 male and 10 female CD-1 mice were fed diets containing 0, 100, 3500 and 7000 ppm Bicyclopyrone for a period of at least 90 Days (0, 15.4/20.8, 542.6/808.5 and 1127.4/ 1343.5 mg/kg/day [M/F]). Mice were regularly monitored for signs of ill health or reaction to the diet. Body weights and food consumption were measured and recorded at pre-determined weekly intervals. Haematology parameters were measured, and all animals were subjected to a detailed necropsy examination after the completion of treatment. Tissues from all animals in the control and high dose groups were subjected to comprehensive histological evaluation. In addition, the liver was also examined from all animals in the low and intermediate dose groups. Additional samples of the liver were taken for possible genomics and immunohistochemistry analysis.

There were no effects on body weights, food consumption, haematology or ophthalmoscopy at any dose levels up to 7000 ppm. A small increase in the number of lens opacities were noted in treated males, however, due to the finding being seen across all male groups and the

findings being common in this species, the findings were considered to be incidental to treatment. These findings were not seen in females.

At 100 ppm bicyclopyrone, liver weights were higher in females when compared to the control group, but, this finding was not accompanied by any histopathological change and is therefore considered non-adverse at this dose.

In males in the 3500 and 7000 ppm groups liver weight were statistically significantly increased (↑27%-↑38%) and this was accompanied by minimal centrilobular hepatocyte hypertrophy (6/10 males and 1/10 females treated at 3500 ppm and 9/10 males and 4/10 females treated at 7000 ppm).

The NOAEL for both sexes is 7000 ppm (1127.4/ 1343.5 mg/kg/day [M/F]). The LOAEL was not established.

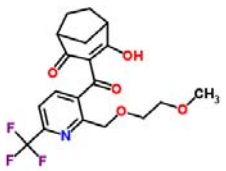
This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirements (OPPTS 870.3100) for a subchronic oral toxicity study in the mice.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS
Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown/beige powder
Lot/Batch number:	SEZ3AP006/Milled
Purity:	94.5%
CAS#:	352010-68-5
Stability of test compound:	Stable

Structure:



Vehicle and/or positive control: Rat and Mouse (modified) No. 1 Diet SQC Expanded (Ground)

Test Animals:

Species	Mouse
Strain	CD-1 (Crl:CD-1(ICR))
Age/weight at dosing	Ordered to be 4 weeks of age Males 27.3 to 37.0 g Females 20.1 to 29.6 g.
Source	Charles River, UK Limited, Margate, Kent
Housing	2 per cage (females) or singly (males) by dose group in suspended polycarbonate cages (overall dimensions 48 x 15 x 13 cm) with stainless steel grid tops and solid bottoms and separate food hoppers
Acclimatisation period	1-2 weeks
Diet	Rat and Mouse (modified) No. 1 Diet SQC Expanded (Ground) *ad libitum*
Water	Mains water *ad libitum*
Environmental conditions	Temperature: 19-23°C Humidity: 37-70% Air changes: Minimum of 15 air changes per hour Photoperiod: 12 hrs dark / 12 hrs light

Study Design and Methods:
In-life dates: Start: 13th March 2007 End: 17th September 2007

Animal assignment: Animals were assigned to treatment groups as below.

Route and duration of administration: The following groups were administered bicyclopyrone in the diet for at least 90 days. The control group received the same diet, without bicyclopyrone.

Table 1: Study design

Test group	Dietary concentration (ppm)	Achieved Doses (mg/kg/day)	# of males	# of females
Control	0	0	10	10
Low	100	15.4/20.8	10	10
Mid	3500	542.6/808.5	10	10
High	7000	1127.4/1343.5	10	10

Diet preparation and analysis:
Formulated diets, at a concentration of 7000 ppm were prepared by direct admixture of the test item to a required amount of untreated diet and blended for 20 minutes in a diet mixer. The 3500 ppm was prepared by adding a measured amount of the 7000 ppm diet to a required amount of untreated diet and blended for 20 minutes in a diet mixer. To prepare the 100 ppm diet, a calculated amount of 3500 ppm diet was added to untreated diet as above to prepare an

intermediate level formulation at a concentration of 500 ppm. The intermediate formulation was then diluted again with untreated diet to prepare the 100 ppm diet.

Blank diet (Rat and mouse (modified) No. 1 Diet SQC Expanded (Ground)) was prepared for Control animals. Diet formulations, in weeks 1-3 were prepared weekly and then frozen at -20°C. Formulations were dispensed 3 times per week and allowed to reach room temperature prior to administration to the animals. Following additional stability testing, from Week 3 formulated diets were prepared and dispensed weekly and stored at room temperature for up to 7 days.

Concentration analysis results:
All analysed formulations were found to be within -7% to +3% of the nominal concentration, indicating acceptable accuracy of formulation.

Homogeneity results:
The coefficient of variance was low (8.7% or below) indicating satisfactory homogeneity of diet formulations.

Stability results:
Stability up to 7000 ppm was satisfactory for 15 days when stored at ambient temperature and protected from light, or when frozen at -20°C.

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable, provided that the cited stability study did indicate that the test compound was stable under conditions of the study.

Observations:
All animals were checked early morning and as late as possible each day for viability.
Once per week, all animals received a detailed clinical examination, including appearance, movement and behaviour patterns, skin and hair condition, eyes and mucous membranes, respiration and excreta.

Bodyweight:
Body weights were recorded weekly, commencing from Pretrial until the completion of treatment.

Food consumption and test substance intake:
The quantity of food consumed by each cage of animals was measured throughout the study and calculated weekly commencing Pretrial until the end of treatment.

Food utilisation was calculated for weeks 1-4, 5-8, 9-13 and 1-13 according to the following formula:

(Cage mean weight gain x 100) / cage total food consumption

The amount of experimental diet ingested was calculated at regular intervals during treatment using the following formula:

$$\text{Achieved intake (g/kg/day)} = \frac{\text{Concentration (p.p.m) x Food Consumption (g/day)}}{\text{Mid-point Body Weight}}$$

Water Consumption:

Water consumption was qualitatively monitored by visual inspection of the water bottles on a weekly basis throughout the study.

Ophthalmoscopic examination:
The eyes of all animals were examined pretrial and week 13 of treatment using an indirect ophthalmoscope after the application of a mydriatic agent (1% Tropicamide, Mydriacyl®). The cornea, anterior chamber, iris, lens, posterior chamber, retina and vessels and optic disc were evaluated.

Haematology:
Blood samples for haematology were obtained from all animals in random order *via* the orbital sinus under isoflurane anaesthesia prior to terminal kill. The animals were not deprived of food overnight prior to sampling.

Approximately 0.35 mL of whole blood was transferred into tubes containing EDTA for haematology investigations.

The parameters for haematology were as follows:

Haemoglobin	Reticulocytes
Red blood cell count	Differential white blood cell count
Haematocrit	Neutrophils
White blood cell count	Lymphocytes
Mean cell volume	Monocytes
Mean cell haemoglobin	Eosinophils
Mean cell haemoglobin concentration	Basophils
Platelets	Large unclassified cells

Clinical chemistry: Not conducted.

Urinalysis: Not conducted.

Investigations *post mortem:*
After at least 90 days of treatment all animals were killed in random order by exposure to carbon dioxide and had their terminal body weight recorded followed by exsanguination.

Macroscopic examination: All animals were examined *post mortem*. The necropsy consisted of a complete external and internal examination, which included orifices (ears, nostrils, mouth, anus, vulva) and cranial, thoracic and abdominal cavities. All gross lesions were recorded in terms of location(s), size (in mm), shape, colour, consistency and number.

Organ weights: From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

Adrenal glands	Ovaries
Brain	Spleen
Epididymides	Testes
Heart	Thymus
Kidneys	Uterus (with cervix and oviduct)

Liver + gall bladder

Paired organs were weighed together.

Tissue submission: The following tissues were examined *in situ*, removed and examined and fixed in an appropriate fixative:

Abnormal tissue
Adrenal gland x 2
Aortic arch
Brain
Epididymis
Eye x 2
Femoral Bone (Including stifle joint)
Gastro-Intestinal tract:
- Stomach
- Duodenum
- Jejunum
- Ileum
- Caecum
- Colon
- Rectum

Harderian gland
Heart
Implant(s)
Kidney x 2
Larynx
Liver + Gall bladder
Lung
Marrow smear (femur)
Mesenteric lymph node
Nasal Cavity
Oesophagus
Optic nerve x 2
Ovary x 2
Pancreas
Pharynx
Pituitary
Preputial/Clitoral Gland
Prostate
Sciatic nerve
Seminal vesicle
Skin + Mammary gland
Spinal Cord
Spleen
Sternum
Submandibular lymph node
Submaxillary (Mandibular) Salivary Gland
Testis x 2
Thigh muscle
Thymus
Thyroid with Parathyroid x 2
Tongue
Trachea
Urinary bladder
Uterus (with Cervix and Oviduct)
Vagina

Microscopic examination: All processed tissues were examined by light microscopy.

Statistics:

Body weight, cumulative weekly body weight gain, food consumption, food utilisation and haematology data were analysed using a parametric ANOVA and pairwise comparisons made using the Dunnett's t-test distribution. If the variances were heterogeneous, log or square root transformations were used in an attempt to stabilise the variances. If the variances remained heterogeneous, log or square root transformations were made in an attempt to stabilise the variances. Organ weights were analysed as above and by analysis of covariance (ANCOVA) using terminal body weight as covariate.

Analyses of variance and covariance were carried out using the MIXED procedure in SAS (9.1.3). Least-squares means for each group were calculated using the LSMEAN option in SAS PROC MIXED. Unbiased estimates of differences from control were provided by the difference between each treatment group least-squares mean and the control group leastsquares mean. Differences from control were tested statistically by comparing each treatment group least-squares mean with the control group least-squares mean using a twosided Dunnett's t-test, based on the error mean square in the analysis. All statistical tests were two sided.

Summary statistics (mean, standard deviation and number of observations) and individual values were presented for organ weights as a percentage of body weight. However, statistical comparisons to control values were not performed for relative organ weight (%), because the ANCOVA results with terminal body weight as covariate provide a more robust statistical determination of this parameter.

The following pairwise comparisons were performed:

Control *vs* Low Dose
Control *vs* Intermediate Dose
Control *vs* High Dose

Histopathology data were analysed using Fishers Exact Probability Test. Histological findings with multiple severities were also analysed using the Mann-Whitney U test. With the exception of the histopathology data, all statistical tests were two-sided and performed at the 5% and 1% significance levels using in-house software. Males and females were analysed separately. Analysis of histopathology data was performed at 5%, 1% and 0.1% significance levels

RESULTS AND DISCUSSION

Mortality:
There were no premature decedents during this study.

Clinical observations:
There were no clinical signs observed which could be attributed to treatment.

Bodyweight and weight gain:
There were no statistically significant differences in absolute body weight. See table 2.

Body weight change was statistically significantly lower in females in the 100 and 7000 ppm groups than in controls, during week 1. These differences were no longer evident as the study progressed and are considered not to be of toxicological significance.

Table 2: Intergroup comparison of bodyweights

	Dietary Concentration of Bicyclopyrone (ppm)							
	Males				Females			
week	0	100	3500	7000	0	100	3500	7000
0	31.7 ± 1.6	30.8 ± 2.0	31.2 ± 2.9	31.9 ± 1.8	23.9 ± 0.9	24.4 ± 2.2	23.5 ± 2.7	23.7 ± 0.9
1	33.3 ± 1.7	32.2 ± 2.7	32.6 ± 3.7	32.8 ± 1.3	25.8 ± 1.1	25.1 ± 2.2	24.6 ± 2.6	24.6 ± 1.2
2	34.5 ± 1.8	34.5 ± 3.1	34.4 ± 4.4	35.0 ± 1.9	26.7 ± 0.9	26.3 ± 2.8	25.4 ± 3.5	25.4 ± 1.5
3	35.7 ± 1.8	36.0 ± 3.2	35.4 ± 4.4	35.2 ± 1.8	27.0 ± 0.8	26.8 ± 3.0	25.4 ± 3.6	26.5 ± 1.5
4	37.7 ± 1.7	37.7 ± 3.8	36.6 ± 4.7	36.9 ± 2.1	27.2 ± 1.0	27.5 ± 3.4	26.6 ± 3.5	26.6 ± 1.7
5	38.6 ± 1.9	38.3 ± 3.9	38.0 ± 5.0	38.5 ± 2.3	28.1 ± 1.5	27.8 ± 3.9	26.8 ± 3.2	27.3 ± 2.0
6	40.0 ± 2.1	39.8 ± 4.2	39.4 ± 5.1	39.5 ± 2.7	28.9 ± 1.9	29.2 ± 4.3	27.3 ± 3.7	27.5 ± 2.4
7	40.7 ± 2.3	41.0 ± 4.6	41.0 ± 5.1	40.0 ± 2.7	29.6 ± 1.7	29.3 ± 4.3	27.4 ± 3.5	28.1 ± 2.1
8	42.2 ± 2.5	42.0 ± 5.2	41.4 ± 5.7	41.4 ± 2.8	30.2 ± 1.7	29.8 ± 4.9	28.4 ± 4.0	28.4 ± 2.1
9	43.2 ± 2.8	43.7 ± 5.5	42.4 ± 6.3	42.3 ± 3.2	30.4 ± 1.5	31.3 ± 6.0	28.4 ± 4.0	29.2 ± 2.1
10	44.0 ± 2.7	43.8 ± 5.1	42.7 ± 6.6	42.8 ± 3.6	30.9 ± 2.4	31.7 ± 6.1	29.0 ± 3.4	30.0 ± 3.2
11	44.8 ± 3.1	44.8 ± 5.6	43.6 ± 7.0	43.1 ± 4.0	31.0 ± 2.7	33.0 ± 6.2	29.9 ± 3.8	30.8 ± 2.5
12	45.3 ± 3.2	45.6 ± 6.2	44.6 ± 7.2	44.0 ± 4.8	33.0 ± 3.1	33.1 ± 7.5	29.2 ± 4.1	30.2 ± 3.3
13	45.2 ± 3.2	45.3 ± 6.2	44.7 ± 7.1	44.3 ± 4.5	31.6 ± 2.5	33.3 ± 7.6	29.5 ± 4.1	30.7 ± 2.9

Data were taken from pages 34-37 of the study report

Food consumption and compound intake:
Isolated incidences of statistically significant increases in food consumption were noted throughout the study in males treated with 7000 ppm, along with one significantly increased value in females treated with 3500 ppm. These were considered not to be related to treatment due to their minor isolated nature, and lack of a dose relationship.

Water Consumption
Visual inspection indicated no observable differences throughout the treatment period.

Ophthalmoscopic examination:
A small increase in the number of lens opacities were noted in the treated males, however, due to the finding being seen across all male groups (including the concurrent control group)

and the findings being common in this species the findings were considered to be incidental to treatment. These findings were not seen in females.

Haematology:
There were no differences seen which were considered to be related to treatment.
Higher mean cell haemoglobin concentration was seen in males treated with 3500 ppm and higher monocytes in animals treated with 100 ppm, compared to their control, were considered to be unrelated to treatment in the absence of similar findings in the 7000 ppm group.

Sacrifice and pathology:

Organ weights:

Dose related statistically significant increases in absolute and adjusted liver weights were noted in males at both 3500 and 7000 ppm. Absolute weights were 26 and 38% higher than controls in the 3500 and 7000 ppm groups, respectively. Adjusted weights were 24 and 35% higher than controls in the 3500 and 7000 ppm groups, respectively. There were no statistically significant changes in absolute liver weights in females, however, there were statistically significant increases in adjusted liver weights in all treated female groups. See table 3.

Higher adjusted adrenal gland weights in females in the 100 ppm group compared to their control were considered not to be related to treatment in the absence of a similar finding in the higher dose groups.

Table 3: Absolute and adjusted liver weights, group mean values

	Dietary Concentration of Bicyclopyrone (ppm)							
	Males				Females			
	0	100	3500	7000	0	100	3500	7000
Liver (abs.)	2.23 ± 0.21	2.45 ± 0.28	2.83[b] ± 0.48 (↑27%)	3.07[b] ± 0.43 (↑38%)	1.76 ± 0.26	2.16 ± 0.50	1.96 ± 0.35	2.04 ± 0.32
Liver (adj.)	2.28 ± 0.08	2.40 ± 0.07	2.81[b] ± 0.07 (↑24%)	3.09[b] ± 0.07 (↑35%)	1.75 ± 0.06	2.00[a] ± 0.06 (↑14%)	2.08[b] ± 0.06 (↑19%)	2.09[b] ± 0.06 (↑19%)

Data were taken from pages 50-51 of the study report
[a] = $p<0.05$, [b] = $p<0.01$

Macroscopic findings:
All necropsy findings were typical of spontaneously arising background findings in mice of this strain and age, for this kind of study at Charles River Laboratories Preclinical Services, Edinburgh.

Microscopic findings:
Minimal centrilobular hepatocyte hypertrophy was seen in the livers of 6/10 males and 1/10 females treated at 3500 ppm and 9/10 males and 4/10 females treated at 7000 ppm. There was no evidence of centrilobular hepatocyte hypertrophy in mice in the 100 ppm dose group. This increased incidence of hepatocyte hypertrophy along with the changes in liver enzymes

were considered adaptive and not adverse.There were other minor liver changes which showed no dose response including focal necrosis, inflammatory cell foci, and centrilobular glycogen vacuolization. See table 4.

In two males at 7000 pm, there were optical findings (animal 33- minimal acute periorbital inflammation, animal 35-retinal fold). Retinal fold was also observed in a single control female. All other findings were typical of spontaneously arising background findings in mice of this strain and age, on this kind of study at Charles River Laboratories Preclinical Services Edinburgh.

Table 4: Microscopic Liver changes

	Dietary Concentration of Bicyclopyrone (ppm)							
	Males				Females			
	0	**100**	**3500**	**7000**	**0**	**100**	**3500**	**7000**
No. of animals	10	10	10	10	10	10	10	10
Centrilobular Hepatocyte Hypertrophy (minimal)	0	0	6* (60%)	9*** (90%)	0	0	1 (10%)	4 (40%)

Data were taken from pages 78 of the study report
Significantly different from the controld:* P < 0.05, **P < 0.01, *** P < 0.001

INVESTIGATOR'S CONCLUSIONS:
Dietary administration of 3500 and 7000 ppm bicyclopyrone to mice for at least 90 days was associated with minimal centrilobular hepatocyte hypertrophy and an increase in liver weight in males.

REVIEWER COMMENTS:

The NOAEL for both sexes is 7000 ppm (1127.4/ 1343.5 mg/kg/day [M/F]). The LOAEL was not established.

In the absence of the clinical chemical analyses, the APVMA/OCS (Austrailia) thinks the effects in the liver (organ weight change with histopathological change) cannot be ruled out as non-adverse. The OCS therefore considers the NOAEL to be 100 ppm for the effects seen in both sexes at ≥3500 ppm.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirements (OPPTS 870.3100) for a subchronic oral toxicity study in the mice, although it should be noted that standard clinical chemistry and urinalysis paremeters were not conducted in this study. EPA and PMRA (Canada) agree on the NOAEL/LOAEL for this study. All three agencies agree on the regulatory classification of this study.

(Shearer J & Wood M, 2009)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anw Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

STUDY TYPE: 90-Day Oral Toxicity [feeding]-[rat]; OPPTS 870.3100 [§82-1a] (rodent); OECD 408.

PC CODE: 018986 **DP BARCODE:** D425155

TEST MATERIAL (PURITY): NOA449280 (94.5%)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Shearer J & Roberson B, 2009. NOA449280 - 13 Week Rat Dietary Toxicity Study. Charles River, Tranent, Edinburgh, EH33 2NE, UK. Laboratory Report No. 28457. 24/04/2009. Unpublished. (Syngenta File No. NOA449280_11003) MRID 47841975

SPONSOR: Syngenta Limited, Product Safety, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

JUSTIFICATION OF THE TEST SYSTEM
The rat was selected as the test model as at this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. The dietary route of administration was selected for this study as this route was defined by the Sponsor as a possible route of human exposure.

EXECUTIVE SUMMARY

In a subchronic toxicity study in rats (MRID #47841975), groups of 10 male and 10 female Han Wistar rats were fed diets containing 0, 2.5, 10, 2500 or 5000 ppm (0, 0.18/0.22, 0.72/0.88, 183.07/228.82 and 363.12/442.26 mg/kg/day [M/F]) bicyclopyrone (NOA449280, 94.5%) for a period of at least 90 days.

The animals were monitored regularly for viability and for signs of ill health or reaction to treatment. Detailed functional observations were performed once during treatment over a two week period. Body weights and food consumption were measured and recorded at predetermined intervals from pretrial up until the completion of treatment. Ophthalmic assessments were undertaken on all animals 3 times throughout the study. Blood and urine samples were also collected, during the last week of treatment, for laboratory investigations. All animals were subjected to a detailed necropsy examination after the completion of

treatment. Tissues from all animals in the control and high dose groups and the eyes from all groups were subjected to a comprehensive histological evaluation. Additional liver tissue from all animals was taken and stored for possible investigative studies.

The effects are as follows:

There were no effects due to treatment with 2.5 and 10 ppm bicyclopyrone.

At 2500 ppm bicyclopyrone, 50% of males and 70% of females had at least one opaque eye. Group mean body weights were statistically significantly reduced in males when compared to their control (↓10-↓12%). Slightly lower food consumption was noted in males. Following covariate analysis, kidney weights were statistically significantly higher than control in males (↑15%). Absolute kidney weights were not changed. Minimal to moderate keratitis was observed in males (30%) and females (40%) compared to the controls (0%).

At 5000 ppm bicyclopyrone, 40% of males and 60% of females had at least one opaque eye. Group mean body weights were statistically significantly reduced in males when compared to their control (↓7-14%). Slightly lower food consumption, with isolated statistical significance, was noted in males (↓11-↓17%). Following covariate analysis, kidney weights in males were statistically significantly higher than controls (↑14%). Absolute kidney weights were not changed. Minimal to moderate keratitis was observed in males (40%) and females (30%) compared to the controls (0%).

Based upon the effects in this study, the LOAEL is 2500 ppm (183.07/228.82 mg/kg/day [M/F]) based upon an increased incidence of eye opacity in both sexes, decreased absolute body weights, decreased food consumption in males, and an increased incidence of ocular keratitis in both sexes. The NOAEL is 10 ppm (0.72/0.88 mg/kg/day [M/F]).

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirements (OPPTS 870.3100) for a subchronic oral toxicity study in the rat.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown/beige powder
Lot/Batch number:	SEZ3AP006
Purity:	94.5%
CAS#:	352010-68-5
Stability of test compound:	Stable
Structure:	

Vehicle and/or positive control: Rat and mouse (modified) No. 1 Diet SQC Expanded (Ground) supplied by Special Diets Services Limited, 1 Stepfield, Witham, Essex

Test Animals:

Species	Rat
Strain	HanWistar (CrL: WI(Han))
Age/weight at dosing	7-8 weeks/Males 194 to 253 g, Females 124 to 166 g
Source	Charles River UK Limited, Margate, Kent
Housing	2 per cage by sex and dose group
Acclimatisation period	24 days
Diet	Rat and Mouse (modified) No. 1 Diet SQC Expanded (Ground) *ad libitum,* except during motor activity assessment and urine collection.
Water	mains water *ad libitum*, except during motor activity assessment and urine collection.
Environmental conditions	Temperature: 19-23°C Humidity: 40-70% Air changes: 15/hour Photoperiod: Light hours 0700-1900h.

Study Design and Methods:
In-life dates: Start: 7th December 2006 End: 18th June 2007

Animal assignment: Fifty males and 50 females were assigned to the study. Two remaining animals of each sex were assigned as extra animals.

Route and duration of administration: The following groups were administered bicyclopyrone in the diet for at least 90 days. The control group received the same diet, without bicyclopyrone.

Table 1: Study design

Test group	Dietary concentration (ppm)	# male	# female
1 Control	0	10	10
2 Low	2.5	10	10
3 Intermediate I	10	10	10
4 Intermediate II	2500	10	10
5 High	5000	10	10

Table was taken from page 19 of the study report

Diet preparation and analysis:
Formulated diet at a concentration of 5000 ppm was prepared by direct admixture of the test item to a required amount of untreated diet and blended for 20 min in a diet mixer. The 2500 ppm diet was prepared by taking the required amount of 5000 ppm diet and adding it to a measured amount of blank diet and blended for 20 min in a diet mixer. The 10 and 2.5 ppm diet were prepared by serial dilution of the 2500 ppm using an intermediate level formulation of 250 ppm. This was done by adding the required amount of formulated diet to a calculated amount of blank diet and blended in a mixer for 20 min. Diet formulations were prepared on a weekly basis and stored frozen at -20°C. Formulations were dispensed 3 times per week and allowed to reach room temperature prior to administration to the animals.

Prior to study commencement, trial formulations of the lowest and highest anticipated concentrations were analyzed for stability and homogeneity by the Sponsor.

Concentration analysis results: The majority of analyzed concentrations were generally found to be within ca ±9% of the nominal concentration, indicating acceptable accuracy of formulation. However during Week 12, the concentrations of diet formulations from animals treated at 2.5 and 5000 ppm were found to be -10.8 and -24.8%, respectively. After reanalysis, the formulation at 5000 ppm was found to be +8.8%, however, the formulation at 2.5 ppm was still found to be -10.8%. As the results after reanalysis were either within or just slightly outside the acceptable range and that all other analyses were within ±10% these diet analyses were considered not to affect the outcome or integrity of the study.

Homogeneity results: The coefficients of variation of the diet prepared after analysis/reanalysis were within 10% indicating acceptable homogeneity.

Stability results: Prior to study commencement trial formulations of the lowest and highest anticipated concentrations were analyzed for stability and homogeneity by the Sponsor. This work was then transferred and carried out in the Toxicology Support Laboratories of Charles River under a separate protocol (Charles River Study No. 423884, Method No. 2388), where formulated diets in the concentration range of 2.5-7000 ppm were analyzed for stability, concentration and homogeneity. The study data indicate that diets were stable for 2-3 months.

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable, provided that the cited stability study did indicate that the test compound was stable under conditions of the study.

Observations
All animals were checked early morning and as late as possible each day for viability.

Once per week, all animals received a clinical examination, including appearance, movement and behavior patterns, skin and hair condition, eyes and mucous membranes, respiration and excreta.

Bodyweight: Body weights were recorded weekly.

Food consumption and test substance intake:
The quantity of food consumed by each cage of animals was measured and recorded weekly

The amount of experimental diet ingested was calculated at regular intervals during treatment using the following formula:

$$\text{Achieved intake (mg/kg/day)} = \frac{\text{Concentration (p.p.m) x Food Consumption (g/rat/day)*}}{\text{Mid-point Body Weight**}}$$

*Food consumption was calculated per animal using a cage mean value over a 7 day period
**The mid-point body weight is an average of the body weights at the start and end of each period for which food consumption was measured.

Water Consumption:
Water consumption was monitored weekly by qualitative visual inspection of the water bottles.

Ophthalmoscopic examination:
An ophthalmoscopic examination was carried out on all animals during Pretrial and Weeks 6/7 and 13 of treatment. The eyes were examined using an indirect ophthalmoscope after the application of a mydriatic agent (1% Tropicamide, Mydriacyl®). The following areas were examined: Cornea, anterior chamber, iris, lens, posterior chamber, retina and vessels and optic disc.

Functional Observation Battery (FOB):
Once during the treatment period (Week 12/13) cageside and arena observations and functional tests were carried out on all animals.

Cageside observations
Posture/condition on first approach (animal undisturbed), checking for:

Prostration
Lethargy
Writhing
Circling
Breathing abnormalities
Gait abnormalities
Tremor
Fasciculation
Convulsions
Biting (of cage components or self-mutilation)
Vocalizations
Piloerection
Ease of removal from cage

Body temperature:

This was taken and recorded from the implanted electronic identification chip.

Condition of the eyes, checking for:
Pupillary function
Miosis
Mydriasis
Exophthalmos
Encrustation
Lacrimation

Condition of coat.
Presence of salivation.
Overall ease of handling.

Observations in a standardized arena (2 min observation period):
Latency (time to first locomotory movement)
Level of mobility
Rearing
Grooming
Urination/defecation
Arousal (level of alertness)
Posture, tremor/convulsions, vocalization, piloerection – recorded as for cageside observations
Palpebral closure
Gait abnormalities
Stereotypy (excessive repetition of behaviors) and/or unusual behaviors

Functional Tests
Reaction to sound (click above head)
Reaction to touch on the rump with a blunt probe

Grip strength: A strain gauge was used, to which was attached a wire pull bar. Once the animal had gripped the bar, the body was pulled until its grasp was broken; the strain gauge recorded the force required. The procedure was carried out three times for the fore and hind limbs, and the mean grip strengths calculated.

Pain perception: This was assessed by measurement of the tail flick response, using a technique based on the method devised by D'Amour and Smith (1941). The apparatus used shone a calibrated infra-red heat source onto the tail.

Landing foot splay: Maize oil was applied to the hind paws of each animal. The animal was then held in a horizontal, prone position with the nose *ca* 30 cm above a bench surface covered with absorbent paper. When the animal was calm, it was dropped. The distance between the prints of the central footpads was measured. The procedure was repeated 3 times and the mean foot splay calculated. If the rat did not land properly on its feet, this was recorded. In the protocol it stated that animals would be dropped from a height of *ca* 32 cm, however, the technicians followed the SOP which stated *ca* 30 cm in error. This deviation was considered not to have affected the outcome or integrity of the study.

Motor activity (recorded at approximately standardized time of day): Each animal was placed in an individual monitoring cage, scanned by a motion sensor utilizing infra-red pyroelectric detectors. Movement is detected in 3 dimensions anywhere in the cage, and is differentiated into large and small movements. Each animal was monitored for one session of 1 hour, activity counts being recorded over successive periods of 5 min each.

Haematology and clinical chemistry:
Blood samples for haematology (0.5 mL, into EDTA tubes), clinical chemistry (1.5 mL, into lithium heparin tubes) and coagulation (0.45 mL, into 0.05 mL trisodium citrate tubes) were obtained from all animals in a random order *via* the orbital sinus under isoflurane anesthesia prior to terminal kill. The animals were not deprived of food overnight prior to sampling.

The parameters for haematology, coagulation and clinical chemistry parameters were as follows:

Haemotology parameters:
haemoglobin
haematocrit
red blood cell count
White blood cell count
Mean cell volume
Mean cell haemoglobin
Mean cell haemoglobin concentration
Platelets
Reticulocytes
Differential white blood cell counts:
- Neutrophils
- Lymphocytes
- Monocytes
- Eosinophils
- Basophils
- Large unclassified cells

Clinical chemistry parameters:

Urea	Total protein
Glucose	Albumin
Aspartate Aminotransferase	Globulin – derived
Alanine Aminotransferase	AG ratio – derived
Alkaline Phosphatase	Cholesterol
Glutamate Dehydrogenase	Triglycerides
Sodium	Creatinine
Potassium	Creatinine Kinase
Chloride	Total Bilirubin
Calcium	Phosphate

Coagulation Parameters

Prothrombin time
Activated Partial Thromboplastin Time

Urinalysis:
Urine samples were collected over an approximate 4 hour period from all animals during Week 13. Animals were housed individually in metabolism cages and were deprived of food and water throughout the collection period.

The parameters for urinalysis were as follows

Volume
Specific Gravity
Colour
pH
Protein
Glucose
Ketones
Urobilinogen
Bilirubin
Blood Pigments
Microscopy of the Spun Deposit

Investigations *post mortem:*
After at least 90 days of treatment, all animals were killed in a randomised order by exposure to carbon dioxide and had their terminal body weight recorded, followed by exsanguination.

Macroscopic examination:
The necropsy consisted of a complete external and internal examination, which included orifices (ears, nostrils, mouth, anus, vulva) and cranial, thoracic and abdominal cavities. All gross lesions were recorded in terms of location(s), size (in mm), shape, color, consistency and number.

Organ weights: From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

Adrenal glands
Brain
Epididymides
Heart
Kidneys
Liver
Ovaries
Spleen
Testes
Thymus
Uterus

Paired organs were weighed together.

Tissue examination: The following tissues were examined *in situ*, removed and examined and fixed in an appropriate fixative:

Abnormal tissue
Adrenal glands
Aortic arch
Brain
Epididymides
Eyes
Pharynx
Pituitary
Prostate
Rib
Sciatic Nerve
Seminal Vesicles

Gastro-intestinal Tract
- Stomach
- Duodenum
- Jejunum
- Ileum
- Caecum
- Colon
- Rectum

Harderian Glands
Heart
Implant
Kidneys
Larynx
Liver
Lung
Marrow Smear (Femur)
Mesenteric Lymph Node
Nasal Cavity
Oesophagus
Optic Nerves
Ovaries
Oviducts
Pancreas
Skin + Mammary Gland
Spinal Cord
Spleen
Sternum
Submandibular Lymph Node
Submaxillary (Mandibular) Salivary Glands
Testes
Thigh Muscle
Thymus
Thyroid with Parathyroid (x2)
Tongue
Trachea
Urinary Bladder
Uterus
Vagina

Microscopic examination: All processed tissues (except implant and rib) were examined by light microscopy.

A blood film smear was made from all EDTA hematology samples and stained for possible examination. Femoral bone marrow smears were also taken at necropsy and stained using Romanowsky stains for possible examination. Both smears and stains were not evaluated as it was considered that examination would not yield any further information.

Statistics:
Cumulative weekly body weight gain, body weight, food consumption, food efficiency, haematology, coagulation, clinical chemistry, selected urinalysis, motor activity and quantitative FOB measurement data were analyzed using a parametric ANOVA and pairwise comparisons made using the Dunnett's t-test distribution. If the variances were heterogeneous, log or square root transformations were used in an attempt to stabilize the variances. Organ weights were analyzed as above and by analysis of covariance (ANCOVA) using terminal body weight as covariate.

Summary statistics (mean, standard deviation and number of observations) and individual values were presented for organ weights as a percentage of body weight. However, statistical comparisons to control values were not performed for relative organ weight (%), because the ANCOVA results with terminal body weight as covariate provide a more robust statistical determination of this parameter.

The following pairwise comparisons were performed:
Control *vs* Low Dose

Control *vs* Intermediate I Dose
Control *vs* Intermediate II Dose
Control *vs* High Dose

Histopathology data were analysed using Fishers Exact Probability Test. Histological findings with multiple severities were also analysed using the Mann-Whitney U test.

All statistical tests were two-sided and performed at the 5% significance level using in-house software. Males and females were analyzed separately.

RESULTS AND DISCUSSION

Mortality: There were no premature decedents during the study.

Clinical observations:

Five males (50%) and seven females (70%) treated at 2500 ppm and four males (40%) and six females (60%) treated at 5000 ppm were noted to have at least one opaque eye. There were no other clinical observations considered to be related to treatment with bicyclopyrone. See table 2.

Table 2. Distribution of opaque eyes (n=10)

Group/sex	Both eyes opaque	One eye opaque
Males- 2500 ppm	2 (20%)	3 (30%)
Males- 5000 ppm	2 (20%)	2 (20%)
Females- 2500 ppm	2 (20%)	5 (50%)
Females – 5000 ppm	2 (20%)	4 (40%)

Data were taken from page 28 of the study report

Bodyweight and weight gain: Absolute body weights and body weight gains are presented in tables 3 and 4. Group mean body weights were generally shown to be statistically significantly reduced in males receiving the 2500 and 5000 ppm diets when compared to their control (↓10-↓12% and ↓7-↓14%, respectively). Group mean body weight gains over the treatment period was generally shown to be statistically significantly reduced in males receiving the 2500 and 5000 ppm diets when compared to their control (↓23-↓26% and ↓26-↓40%, respectively). In females treated at 5000 ppm, body weight gain was noted to be statistically significantly reduced in comparison to their control (↓20-↓55%).

Table 3: Intergroup comparison of absolute bodyweights (g)

	Dietary Concentration of bicyclopyrone (ppm)									
	Male					Female				
Week	0	2.5	10	2500	5000	0	2.5	10	2500	5000
0	229 ± 13	224 ± 16	227 ± 13	229 ± 13	227 ± 5	148 ± 11	146 ± 12	148 ± 9	149 ± 7	153 ± 7
1	269 ± 17	264 ± 21	267 ± 16	260 ± 14	251[a] ± 7 (↓7%)	167 ± 11	166 ± 15	166 ± 9	165 ± 8	162 ± 11
2	300 ± 20	294 ± 27	295 ± 20	284 ± 15	277[a] ± 12 (↓8%)	181 ± 17	180 ± 19	183 ± 11	179 ± 8	177 ± 10
3	326 ± 23	317 ± 34	318 ± 24	303 ± 19	295[a] ± 15 (↓10%)	193 ± 10	194 ± 20	195 ± 12	190 ± 10	189 ± 11
4	348 ±	334 ±	335 ±	318 ± 22	308[b] ± 17	204 ±	204 ±	204 ±	198 ±	193 ±

	27	39	29		(↓11%)	9	22	13	9	11
5	365 ± 29	351 ± 41	351 ± 31	333 ± 25	324[a] ± 19 (↓11%)	213 ± 9	214 ± 23	216 ± 14	209 ± 9	203 ± 13
6	380 ± 30	366 ± 44	365 ± 33	342[a] ± 29 (↓10%)	336[a] ± 22 (↓12%)	218 ± 12	219 ± 24	221 ± 14	212 ± 11	207 ± 11
7	390 ± 30	374 ± 47	372 ± 34	349[a] ± 31 (↓11%)	343[a] ± 22 (↓12%)	224 ± 10	224 ± 23	226 ± 12	217 ± 11	214 ± 12
8	402 ± 29	389 ± 48	385 ± 36	359[a] ± 33 (↓11%)	351[b] ± 23 (↓13%)	228 ± 10	228 ± 24	230 ± 13	220 ± 12	216 ± 11
9	412 ± 30	397 ± 49	392 ± 35	363[a] ± 35 (↓12%)	357[b] ± 25 (↓13%)	232 ± 9	233 ± 26	237 ± 13	226 ± 12	219 ± 10
10	418 ± 31	407 ± 49	398 ± 35	371[a] ± 36 (↓11%)	362[b] ± 26 (↓13%)	232 ± 11	237 ± 27	240 ± 13	226 ± 12	222 ± 11
11	426 ± 30	412 ± 49	403 ± 35	374[b] ± 36 (↓12%)	368[b] ± 26 (↓14%)	239 ± 11	242 ± 26	243 ± 14	231 ± 13	225 ± 10
12	428 ± 30	416 ± 48	406 ± 34	377[b] ± 35 (↓12%)	371[b] ± 26 (↓13%)	242 ± 10	243 ± 26	242 ± 14	231 ± 14	228 ± 10
13	434 ± 31	424 ± 48	415 ± 34	382[b] ± 34 (↓12%)	378[b] ± 26 (↓13%)	240 ± 10	244 ± 28	246 ± 13	234 ± 13	227 ± 10

Data were taken from pages 42 to 45 of the study report
Significantly different from Group 1:[a]=p<0.05, [b]=p<0.01

Table 4: Intergroup comparison of absolute bodyweights gains (g)

	Dietary Concentration of bicyclopyrone (ppm)									
	Male					**Female**				
Days	**0**	**2.5**	**10**	**2500**	**5000**	**0**	**2.5**	**10**	**2500**	**5000**
0-7	40 ± 5	40 ± 7	41 ± 5	31[b] ± 4 (↓23%)	24[b] ± 4 (↓40%)	20 ± 4	21 ± 5	18 ± 5	16 ± 4	9[b] ± 6 (↓55%)
0-14	71 ± 9	70 ± 13	69 ± 10	55[b] ± 8 (↓23%)	50[b] ± 10 (↓30%)	34 ± 12	35 ± 9	35 ± 6	29 ± 5	24[a] ± 6 (↓30%)
0-28	119 ± 15	110 ± 27	108 ± 18	89[b] ± 17 (↓25%)	81[b] ± 15 (↓32%)	57 ± 5	59 ± 11	56 ± 8	49 ± 6	40[b] ± 8 (↓30%)
0-42	151 ± 18	142 ± 32	138 ± 23	113[b] ± 24 (↓25%)	109[b] ± 20 (↓28%)	71 ± 7	73 ± 15	73 ± 10	62 ± 10	53[b] ± 7 (↓25%)
0-84	199 ± 19	192 ± 36	180 ± 24	148[b] ± 29 (↓26%)	144[b] ± 24 (↓28%)	95 ± 10	97 ± 18	94 ± 9	82 ± 12	75[b] ± 6 (↓21%)
0-91	205 ± 20	200 ± 35	189 ± 24	153[b] ± 28 (↓25%)	151[b] ± 24 (↓26%)	93 ± 9	99 ± 20	98 ± 9	85 ± 11	74[b] ± 7 (↓20%)

Data were taken from pages 46 to 49 of the study report
Significantly different from Group 1:[a]=p<0.05, [b]=p<0.01

Food consumption and compound intake:

Food consumption and compound intake data are presented in tables 5 and 6. Slightly lower food consumption, with statistical significance, was noted in males in the 2500 ppm group during the first week only (↓10%) and the 5000 ppm group at numerous observation points (↓11-17%) when compared to their control. Food consumption in females given 5000 ppm was also generally lower than their control, although not statistically significant at any interval. Slightly lower food consumption, which did not achieve statistical significance, was noted in males in the 2.5 and 10 ppm groups. This was unaccompanied by any changes in body weight and was considered to reflect normal variability.

Food efficiency for weeks 1-4 and 1-13 were noted to be significantly reduced in males and females treated with 5000 ppm and males treated at 2500 ppm when compared to their respective controls.

Table 5: Intergroup comparison of food consumption (g/rat/day)

	Dietary Concentration of bicyclopyrone (ppm)									
	Male					Female				
Week	**0**	**2.5**	**10**	**2500**	**5000**	**0**	**2.5**	**10**	**2500**	**5000**
0	-	-	-	-	-	-	-	-	-	-
1	26.6 ± 1.6	25.8 ± 1.8	26.1 ± 1.1	23.9[a] ± 1.5 (↓10%)	22.1[b] ± 1.5 (↓17%)	16.6 ± 0.8	16.8 ± 1.7	16.7 ± 1.6	16.9 ± 0.7	15.3 ± 1.0
2	27.5 ± 1.4	26.4 ± 2.3	26.2 ± 2.7	25.0 ± 1.5	24.6± 1.5	19.4 ± 0.9	17.7 ± 1.7	18.3 ± 1.1	18.1 ± 0.8	18.3 ± 1.4
3	27.5 ± 1.4	26.0 ± 2.4	26.7 ± 2.5	25.4± 1.1	25.3 ± 1.8	18.1 ± 0.7	17.9 ± 2.2	18.9 ± 2.0	18.7 ± 1.2	18.5 ± 1.4
4	26.9± 1.1	25.5 ± 2.3	26.0 ± 2.5	24.9± 1.8	23.9 ± 1.5	19.5 ± 0.8	19.6 ± 1.9	18.8 ± 1.4	18.9 ± 1.4	18.0 ± 1.7
5	26.3 ± 0.9	24.9 ± 2.1	25.3 ± 2.9	24.4 ± 1.6	23.0[a] ± 1.5 (↓13%)	19.5 ± 1.1	19.4 ± 1.5	19.5 ± 1.7	19.4 ± 1.0	18.3 ± 1.1
6	27.1 ± 0.7	24.9 ± 2.1	24.9 ± 2.8	24.1 ± 2.6	23.4[a] ± 1.4 (↓14%)	19.1 ± 0.9	18.8 ± 1.5	19.5 ± 0.7	18.8 ± 1.3	17.9 ± 1.5
7	26.1 ± 0.8	25.0 ± 2.0	24.6 ± 2.7	23.1 ± 2.5	23.3 ± 1.5	18.9 ± 0.9	18.5 ± 1.1	19.0 ± 1.3	19.2 ± 1.1	18.0 ± 1.2
8	25.7 ± 0.6	24.9 ± 2.2	23.9 ± 2.3	23.0 ± 2.5	22.0[a] ± 1.1 (↓14%)	18.1 ± 0.8	18.2 ± 1.3	18.3± 1.0	18.6 ± 1.0	17.4 ± 1.0
9	25.3 ± 0.7	24.0 ± 2.0	24.1 ± 3.0	23.5 ± 1.4	22.5 ± 1.4	18.6± 0.5	18.7 ± 1.2	19.3 ± 1.1	19.3 ± 1.0	17.8 ± 1.0
10	24.9 ± 1.3	23.9 ± 1.4	23.8 ± 2.5	23.6 ± 2.0	22.6 ± 0.8	18.3 ± 1.1	19.0 ± 1.8	18.4 ± 0.6	19.1 ± 1.1	18.4 ± 1.3
11	25.0 ± 0.8	24.7 ± 1.3	23.7 ± 2.4	23.0 ± 1.7	22.6 ± 0.8	18.8 ± 0.7	19.0 ± 1.5	19.3 ± 1.2	19.4 ± 1.3	18.4 ± 1.1
12	24.4 ± 0.9	23.7 ± 1.3	22.4 ± 1.4	23.1 ± 1.5	21.8[b] ± 0.8 (↓11%)	18.1 ± 1.0	17.2 ± 1.3	18.0 ± 1.1	18.7 ± 1.4	17.9 ± 1.1
13	25.0 ± 0.9	24.2 ± 0.7	24.0 ± 2.2	23.1 ± 1.0	25.4 ± 6.4	18.0 ± 0.4	18.1 ± 1.4	19.1 ± 1.1	19.1 ± 1.0	17.3 ± 1.0

Data were taken from pages 50-53 of the study report
Significantly different from Group 1:[a]=$p<0.05$, [b]=$p<0.01$

Table 6: Mean Dose Received (mg/kg bw/day)

bicyclopyrone (ppm)	0	2.5	10	2500	5000
Males	0	0.18	0.72	183.07	363.12
Females	0	0.22	0.88	228.82	442.26

Data were taken from pages 34 -37 of the study report

Water Consumption:

Visual inspection indicated no observable differences throughout the treatment period.

Ophthalmoscopic examination:

During the study, animals treated at 2500 or 5000 ppm were noted to have an increased incidence of opacities present in the cornea, commencing from approximately Week 7 through to completion of treatment. Both eyes were equally affected. Corneal opacity was also noted in controls and the 2.5 and 10 ppm dose groups, though frequencies of corneal opacity at 2.5 and 10 ppm were similar to controls during the study.

Motor Activity and FOB Parameters
All groups exhibited similar motor activity during the test period. No changes in detailed clinical observations or functional observation battery parameters were considered treatment-related.

Haematology: There were no differences in haematological parameters which were considered to be related to treatment.

Blood clinical chemistry:
Albumin was statistically significantly higher in males at 5000 ppm compared with controls. The mean value was marginally outside the range of historical control mean values. In view of small magnitude of difference (↑7%) and the lack of any other clinical chemistry changes or any histopathological changes in the liver the differences were considered to be of no toxicological significance.

Total bilirubin was statistically significantly higher in females at 5000 ppm than in controls. The mean value was within the range of historical control mean values and therefore the difference was considered to be incidental to treatment.
Other differences (some statistically significant) were noted, however, due to the magnitude of change, lack of a dose response and no related histological findings they were considered not to be due to administration of bicyclopyrone.

Urinalysis:
An increase in specific gravity achieved statistical significance in all treated males with the exception of animals treated at 10 ppm but was still slightly increased in this group compared with controls.

Urinary pH in males treated at 5000 ppm were noted to be significantly lower; however, due to the small magnitude of change and no other corroborating evidence, this was considered not to be related to treatment with bicyclopyrone.

Sacrifice and pathology:

Organ weights:
Following covariate analysis kidney weights were statistically significantly higher than control in males treated at 2500 and 5000 ppm (↑15% and ↑14% respectively). Following covariate analysis liver weights were statistically significantly higher than control in both sexes in the 2500 ppm group. However this is considered unrelated to treatment in the absence of a similar finding in the high dose group. There was no change in absolute liver and kidney weights compared with controls. See table 7.

Table 7: Kidney and Liver weights (Absolute and Covariance analysis): Group mean values

	Dietary Concentration of bicyclopyrone (ppm)	
	Males	Females

	0	2.5	10	2500	5000	0	2.5	10	2500	5000
Kidneys (Abs.)	2.42 ± 0.16	2.40 ± 0.16	2.52 ± 0.18	2.70 ± 0.24	2.64 ± 0.20	1.54 ± 0.10	1.52 ± 0.13	1.64 ± 0.12	1.59 ± 0.11	1.54 ± 0.08
Kidneys (Cov.)	2.42 ± 0.05	2.33 ± 0.05	2.49 ± 0.05	2.79[b] ± 0.05 (↑15%)	2.76[b] ± 0.05 (↑14%)	1.53 ± 0.03	1.49 ± 0.03	1.61 ± 0.03	1.61 ± 0.03	1.57 ± 0.03
Liver (Abs.)	14.64 ± 1.13	14.11 ± 2.52	14.77 ± 1.46	15.78 ± 2.64	13.85 ± 1.25	8.20 ± 0.63	8.16 ± 0.88	8.69 ± 0.77	8.93 ± 1.36	8.13 ± 0.33
Liver (Cov.)	13.45 ± 0.46	13.42 ± 0.42	14.43 ± 0.41	16.70[b] ± 0.43 (↑24%)	15.03 ± 0.44	8.15 ± 0.23	7.95 ± 0.24	8.50 ± 0.24	9.10[a] ± 0.23 (↑12%)	8.39 ± 0.24

Data were taken from pages 78 to 85 of the study report
Significantly different from Group 1: [a] = $p<0.05$, [b] = $p<0.01$

Macroscopic findings:

Opaque eyes were observed at increased incidence in both males and females treated at 2500 and 5000 ppm. All other necropsy findings were typical of spontaneously arising background findings in rats of this strain and age, on this kind of study at Charles River Edinburgh.

Microscopic findings:

Minimal to moderate keratitis was observed in 3/10 and 4/10 males treated at 2500 and 5000 ppm, respectively. In females this incidence was 4/10 and 3/10 females treated at 2500 and 5000 ppm, respectively. These findings correlated with necropsy findings of opaque eyes. All other histology findings were typical of spontaneously arising background findings in rats of this strain and age, on this kind of study at Charles River Edinburgh. See table 8.

Table 8: Intergroup comparison of the incidence of selected microscopic findings (n=10 per sex/per dose)

Finding	Dietary Concentration of bicyclpyrone (ppm)									
	Males					Females				
	0	2.5	10	2500	5000	0	2.5	10	2500	5000
Eye: Keratitis	0	0	0	3 (30%)	4 (40%)	0	0	0	4 (40%)	3 (30%)

Data were taken from page 105 of the study report

INVESTIGATORS'S CONCLUSIONS:

The dietary administration of bicyclopyrone to rats for at least 90 consecutive days resulted in a decrease in group mean body weight in males at doses of 2500 and 5000 ppm and a reduction in bodyweight gain in females treated at 5000 ppm.

Opaque eyes, opacities, an absent pupillary reflex in the cornea and keratitis seen at 2500 and 5000 ppm in both sexes were considered to be related to treatment. The No Observed Effect Level (NOEL) for this study is 10 ppm in males and females (equivalent to 0.72 and 0.88 mg/kg body weight/day respectively.

REVIEWER'S COMMENTS:

Based upon the effects in this study, the LOAEL is 2500 ppm (183.07/228.82 mg/kg/day [M/F]) based upon an increased incidence of eye opacity in both sexes, decreased absolute body weights, decreased food consumption in males, and an increased incidence of ocular keratitis in both sexes. The NOAEL is 10 ppm (0.72/0.88 mg/kg/day [M/F]).

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirements (OPPTS 870.3100) for a subchronic oral toxicity study in the rat. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Shearer J & Robertson B, 2009)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar J. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) Date: 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) Date: 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: 13-Week Oral (Capsule) Toxicity study in Beagle Dog. OECD 409 (1998): OPPTS 870.3150 (1998); Directive 88/302/EEC B.30 (1988)

TEST MATERIAL (PURITY): NOA449280 (94.5% w/w)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Jackson A.M, 2009. NOA449280 – 13-Week Oral (Capsule) Toxicity Study in the Beagle Dog. Harlan Laboratories Ltd., (former RCC Ltd), Zelgliweg 1, 4452 Itingen / Switzerland. Laboratory Report No. B18922. 03 November 2009. Unpublished. (Syngenta File No. NOA449280_11051) MRID 47841976

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of this study.

EXECUTIVE SUMMARY

In a subchronic oral toxicity study in dogs (MRID #47841976), groups of four male and four female beagle dogs were dosed orally by capsule with bicyclopyrone (NOA449280) at 0, 5, 25 and 125 mg/kg/day for a period of 13 weeks. Clinical signs, body weight and food consumption were recorded throughout the study. Ophthalmoscopy and veterinary examinations were performed and blood and urine samples were collected for clinical laboratory investigations. Following completion of the scheduled treatment period, a detailed necropsy was performed on all animals and various organs were weighed. A full set of tissues and organs were prepared and examined histopathologically.

All animals survived the scheduled treatment period. No clinical signs were observed that were considered to be related to treatment with the test item. There were no effects on food consumption and body weights which were considered to be related to treatment with the test item. Haematology parameters were unaffected by treatment with the test item. There were increased liver weights in females at the highest dose tested but there were no corroborating

macroscopic or microscopic changes. There were no macroscopic findings in any organ which were considered to be related to treatment with the test item. Microscopically, an increased incidence of inflammation/fibrosis/apoptosis of the pancreas was observed in all treated male groups. These findings were mild in severity, and are likely to be transient as they were not observed in the chronic dog study.

Based upon the effects in this study, the NOAEL is 125 mg/kg bw/day. The LOAEL was not observed.

This 90-day oral toxicity study in the dog is totally reliable (**acceptable/guideline**) and satisfies the guideline requirement for a 90-day oral toxicity study (OPPTS 870.3150; OECD 409) in dogs.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown beige powder
Lot/Batch Number:	SEZ3AP006/MILLED
Purity:	94.5%
Stability of test compound:	Reanalysis date: March 2011
Structures:	

Test animals:	
Species:	Dog
Strain:	Beagle Dog
Age/Weight at dosing:	5.5-7months, 5.8 to 9.8 kg
Source:	Harlan Laboratories Ltd, Laboratory Animal Services, 4414 Füllinsdorf /Switzerland
Housing:	Animals were housed in either individual pens or group housing with a minimum of 2.0 square meters floor space per dog. Dogs were separated during feeding periods and after dosing to facilitate recording of clinical signs. The animals of each treatment group were housed in adjacent pens.
Acclimatization:	For 3 weeks under test conditions
Diet:	Animals received 350 ± 1 g pelleted standard Kliba3353 dog maintenance diet (Provimi Kliba AG,4303 Kaiseraugst / Switzerland)
Water:	Community tap water was supplied *ad libitum* by an automatic watering system.
Environmental conditions:	Temperature: 20 ± 3 °C Humidity 30 - 70%

Air changes: 10 - 15 air changes per hour
Photoperiod: 12-hour light / 12-hour dark

Study Design and Methods:

In life dates: Start: 02 May 2007 End: 15/16 August 2007

Animal assignment and treatment: After arrival the dogs were weighed and the weights were ranked. They were allocated to groups based on a four by four Latin square. The allocation was checked for the presence of litter mates and these were distributed through the groups.

Table 1: Study Design (animal identifier codes)

Dose level	Group 1 0mg/kg/day	Group 2 5 mg/kg/day*	Group 3 25 mg/kg/day*	Group 4 125 mg/kg/day*
Males animals	1-4	5-8	9-12	13-16
Female animals	17-20	21-24	25-28	29-32

*****Dose levels are expressed in terms of material as supplied.

Route and duration of administration: The following groups were administered bicyclopyrone in gelatin capsules, daily for 13 weeks. The control group received empty capsules.

Dose preparation and analysis: The appropriate amount of test item was weighed directly into gelatin capsules. The individual weights of test item required for daily administration was adjusted based on the most recently recorded body weight.

Observations: Each animal was examined at least twice daily from pretest for any change in behaviour, reaction to treatment or ill-health. A description of any abnormality was recorded from commencement of the pretest period. Additionally, from commencement of pretest period each animal was examined thoroughly outside the pen, weekly.

Body weights: The body weight of each animal was recorded at least once weekly from commencement of the pretest period and before necropsy.

Food consumption: Food consumption was recorded daily from commencement of the pretest period. The daily ration was weighed before and after feeding. Individual values over the week were presented in the report.

Ophthalmoscopic examination and veterinary examination: Each animal was examined at pretest and week 13.

Haematology and clinical chemistry: Blood and urine samples were collected from all animals at pretest, week 1 and week 13. Blood samples were additionally taken in week 4 and week 8. The animals were fasted overnight but allowed access to water *ad libitum*. The samples were collected early in the working day to reduce biological variation caused by circadian rhythms.

Anticoagulants used for blood collection were tri-potassium EDTA (haematology) or sodium citrate, 3.2% at a 9:1 ratio of blood to anticoagulant (coagulation). The following haematology parameters were determined:

Erythrocyte count	Platelet count
Hemoglobin concentration	Reticulocyte count
Hematocrit	Reticulocyte maturity index
Mean corpuscular volume	Total leukocyte count
Red cell volume distribution width	Differential leukocyte count
Mean corpuscular hemoglobin	Coagulation:
Mean corpuscular hemoglobin concentration	Thromboplastin time
Hemoglobin concentration distribution width	Activated partial thromboplastin time

The anticoagulant used for blood collection was lithium heparin. The following clinical biochemistry parameters were determined.

Glucose	Alkaline phosphatase
Urea	Gamma-glutamyl-transferase
Creatinine	Calcium
Bilirubin, total	Phosphorus
Cholesterol, total	Sodium
Triglycerides	Potassium
Aspartate aminotransferase	Chloride
Alanine aminotransferase	Protein, total
Glutamate dehydrogenase	Albumin
Creatine kinase	Globulin
Lactate dehydrogenase	Albumin/Globulin Ratio

Urinalysis: Urine samples were additionally collected in week 6 (in addition to the samples collected at pretest, week 1 and week 13). The samples were collected from the first animal in each group in the order 4, 2, 1, 3 followed by the second animal in this group order until all animals were sampled. The animals were fasted overnight but allowed access to water *ad libitum*. The samples were collected early in the working day to reduce biological variation caused by circadian rhythms. Urine was collected into a specimen vial using a catheter.

The following urinalysis parameters were determined:

Relative density	Glucose
Color	Ketone
Appearance	Urobilinogen
pH	Bilirubin
Nitrite	Erythrocytes
Protein	Leukocytes

Investigations *post mortem:*
All animals were anaesthetised by intravenous injection of sodium pentobarbital and killed by exsanguination at the end of the treatment period.

Macroscopic examination: All animals were examined *post-mortem.* This involved an external observation and an internal examination of all organs and structures.

Organ weight: The following organs were weighed before fixation. Paired organs were weighed separately.

Adrenal gland (l, r)	Ovaries (l, r)
Brain (including brainstem)	Spleen
Epididymides (l, r)	Testes (l, r)

Heart
Kidney (l, r)
Liver
Thymus
Thyroid gland with parathyroid (l, r)
Uterus (with cervix)

Tissue submission: The following tissues were examined *in situ*, removed and examined and fixed to an appropriate fixative:

Adrenal glands
Aorta
Bone - femur including articular surface*
Bone marrow - femur
Brain - including sections of forebrain, upper brain stem, mid brain, medulla oblongata, pons, cerebral and cerebellar cortex
Epididymides (fixed in Bouin's solution)
Esophagus
Eyes with optic nerve (fixed in Davidson solution)
Female and male mammary gland area
Gallbladder
Heart
Kidneys and ureters
Large intestine - cecum, colon, rectum
Larynx
Liver
Lungs with bronchi and bronchioles, infused with formalin
Lymph nodes - retropharyngeal, mesenteric
Nasal cavities (only level 3 of 4)
Oro-nasal pharynx - adjacent to hard palate
Ovaries
Pancreas
Parathyroid gland
Peyer's patches
Pituitary gland
Prostate gland (accessory sex organ)
Salivary glands - mandibular, parotid, sublinguall
Sciatic nerve
Skeletal muscle - semimembranosus, tibialis cranialis, vastus medialis and gastrocnemius
Skin and subcutaneous tissue
Small intestine - duodenum, jejunum, ileum
Spinal cord - cervical (C1), midthoracic (T7) and lumbar (L7) segments including roots and dorsal root ganglia at lumbar levels
Spleen
Stomach
Testes (fixed in Bouin's solution)
Thymus
Thyroid gland
Tongue
Trachea
Urinary bladder
Uterus with cervix and oviducts
Vagina
All gross lesions

* Tissues collected and stored but not examined unless requested by the Sponsor.

Microscopic examination: All organ and tissue samples to be examined by the study pathologist were processed, embedded and cut to a nominal thickness of 4 micrometers and stained with hematoxylin and eosin, then examined by light microscopy.

Statistics: Body weights, body weight gain, food consumption, haematology, clinical biochemistry, urinalysis and organ weights were considered by analysis of variance, separately for males and females.

Organ weights were also considered by analysis of covariance on terminal body weight, separately for males and females. Summary data were presented for organ to body weight ratios but these were not analyzed statistically as the analysis of covariance provides a better method of allowing for differences in terminal body weights.

Analyses of variance and covariance were carried out using the MIXED procedure in SAS (2004). Differences from control were tested statistically by comparing each treatment group mean with the control group mean using Dunnett's test, based on the error mean square in the analysis.

RESULTS AND DISCUSSION

Mortality: All animals survived the scheduled treatment period.

Clinical observations: There were no clinical signs considered to be related to treatment with the test item.

Food consumption: There were no effects on food consumption which were considered to be related to treatment with the test item.

Occasional fluctuations were observed for animals of all groups including the controls, but they did not show a relationship to dose level and were considered to reflect normal biological variation.

Bodyweight: There were no effects on body weights which were considered to be related to treatment with the test item. See table 2.

Table 2: Mean intergroup comparison of absolute bodyweights (kg) by week

	Dietary Concentration of bicyclopyrone (mg/kg/day)							
	Males				Females			
Day/Week	0	5	25	125	0	5	25	125
0	8.50 ± 0.55	8.45 ± 1.10	8.23 ± 0.92	8.03 ± 0.91	7.08 ± 0.98	6.85 ± 1.02	6.53 ± 0.72	7.10 ± 1.43
Week 1	8.73 ± 0.67	8.85 ± 0.99	8.68 ± 0.98	8.60 ± 0.90	7.28 ± 0.97	7.40 ± 0.80	7.08 ± 0.68	7.73 ± 1.31
Week 4	9.30 ± 0.62	9.25 ± 1.21	9.40 ± 0.78	9.13 ± 0.90	7.78 ± 0.86	8.03 ± 0.63	7.60 ± 0.78	8.40 ± 1.40
Week 8	9.95 ± 0.64	9.45 ± 1.44	9.78 ± 0.75	9.33 ± 0.87	8.40 ± 0.98	8.53 ± 0.50	8.28 ± 0.60	8.83 ± 1.31
Week 14	10.50 ± 0.78	10.15 ± 1.58	10.45 ± 0.79	9.78 ± 1.09	8.85 ± 0.97	8.93 ± 0.63	8.87 ± 0.59	9.40 ± 1.25

Data were taken from pages 36-39 of the study report

Ophthalmoscopy: There were no ophthalmic findings considered to be related to treatment with the test item.

There were a small number of common spontaneous findings which are typically seen in dogs of this age and strain in this facility.

Veterinary examination: There were no effects in the veterinary examination which were considered to be related to treatment with the test item.

The findings were of a type commonly seen in animals at these laboratories and the incidence did not show a relationship to dose level.

Haematology: There were minor changes in haematology which were considered to be related to treatment with the test item. See table 2.

Statistically significantly lower values for haemoglobin (↓11% at weeks 8 and 13) and haematocrit (↓10% at week 8 and ↓9% at week 13) in males were noted at 125 mg/kg/day. At 25 m/kg/day, there was a statistically significant decrease in haemoglobin as well (↓11%). These lower values were within the 95th percentile of the historical control mean and concurrent control values for these parameters were at the high end of the historical control range. Therefore, the effects were not considered to be toxicologically adverse.

At week 13, there was a minor statistically significant basophil count at 25 and 125 mg/kg/day in males (↓58 and ↓65%). In treated males there was a flat dose response for decreased thromboplastin times (↓11-17%) at week 13. For 125 mg/kg/day females, there was a minor increase in thromboplastin times at week 8 (↑19%). There were transient increases in the reticulocyte counts in 5 and 125 mg/kg/day females (↑~100%) at weeks 1, 4 and 8, but there were no increases in 25 mg/kg/day.

Lower reticulocyte maturity indices (females), basophil count (males), large unstained cells (males) and thromboplastin time (males) and higher platelet count (both sexes) in dosed animals were all within the 95th percentile of the historical control mean. These changes were therefore considered to be of no or low toxicological significance.

Table 2: Intergroup comparison of haematology results

	Dose Level of bicyclopyrone (mg/kg/day)							
	Males				Females			
Parameter	**0**	**5**	**25**	**125**	**0**	**5**	**25**	**125**
Hemoglobin (mmol/L):								
Pre-test	8.88 ± 0.46	8.93 ± 0.83	8.38 ± 0.51	8.75 ± 0.41	9.10 ± 0.36	9.33 ± 0.67	8.88 ± 0.49	8.43 ± 0.62
Week 1	8.79 ± 0.49	9.31 ± 0.61	8.65 ± 0.47	8.64 ± 0.59	8.96 ± 0.47	9.45 ± 0.52	8.55 ± 0.52	8.75 ± 0.37
Week 4	8.85 ± 0.81	9.15 ± 1.03	8.60 ± 0.55	8.29 ± 0.68	8.83 ± 0.57	8.73 ± 0.81	8.25 ± 0.38	8.19 ± 0.78
Week 8	9.73 ± 0.50	9.43 ± 0.75	8.80* ± 0.26 (↓10%)	8.68* ± 0.17 (↓11%)	9.10 ± 0.59	9.25 ± 0.99	8.90 ± 0.22	8.70 ± 0.66
Week 13	10.08 ± 0.59	9.88 ± 0.43	9.65 ± 0.24	9.00** ± 0.22 (↓11%)	10.15 ± 0.82	9.88 ± 0.64	9.70 ± 0.58	9.31 ± 1.04
Basophil Count (re. l.)								
Pre-test	0.010 ± 0.001	0.011 ± 0.005	0.010 ± 0.006	0.011 ± 0.002	0.010 ± 0.003	0.011 ± 0.004	0.009 ± 0.004	0.008 ± 0.003
Week 1	0.009 ± 0.002	0.012 ± 0.005	0.009 ± 0.001	0.008 ± 0.001	0.011 ± 0.003	0.011 ± 0.004	0.009 ± 0.002	0.009 ± 0.002
Week 4	0.010 ± 0.003	0.012 ± 0.003	0.011 ± 0.006	0.007 ± 0.003	0.013 ± 0.002	0.011 ± 0.005	0.010 ± 0.001	0.007* ± 0.002 (↓46%)
Week 8	0.013 ± 0.004	0.012 ± 0.005	0.011 ± 0.004	0.009 ± 0.001	0.015 ± 0.001	0.012 ± 0.005	0.010 ± 0.002	0.010 ± 0.002
Week 13	0.019 ± 0.006	0.013 ± 0.006	0.008* ± 0.001 (↓58%)	0.007** ± 0.005 (↓66%)	0.027 ± 0.006	0.024 ± 0.013	0.020 ± 0.009	0.016 ± 0.007
Thromboplastin Times (re. l.)								
Pre-test	0.900 ± 0.048	0.810 ± 0.066	0.860 ± 0.052	0.883 ± 0.049	0.840 ± 0.063	0.853 ± 0.046	0.903 ± 0.058	0.860 ± 0.039
Week 1	0.853 ± 0.046	0.815 ± 0.045	0.838 ± 0.046	0.820 ± 0.072	0.798 ± 0.048	0.830 ± 0.047	0.863 ± 0.068	0.833 ± 0.064
Week 4	0.843 ± 0.046	0.791 ± 0.095	0.835 ± 0.034	0.873 ± 0.080	0.768 ± 0.051	0.833 ± 0.069	0.848 ± 0.089	0.870 ± 0.075
Week 8	0.865 ± 0.066	0.815 ± 0.057	0.808 ± 0.055	0.813 ± 0.103	0.740 ± 0.029	0.785 ± 0.047	0.785 ± 0.070	0.883* ± 0.074 (↑19%)
Week 13	0.939 ± 0.068	0.778** ± 0.034 (↓17%)	0.835* ± 0.033 (↓11%)	0.785** ± 0.051 (↓16%)	0.838 ± 0.061	0.865 ± 0.059	0.878 ± 0.069	0.935 ± 0.037

Data were taken from pages 42-70 of the study report

Clinical biochemistry: There were minor changes in clinical chemical parameters. See table 3.

Decreased plasma cholesterol values were recorded in males and females after treatment at 125 mg/kg/day (↓23-62% for males, and ↓36-43% in females). Cholesterol in males at 125 mg/kg/day was 23% lower than concurrent controls, before dosing had commenced, which indicates that these animals may have a predisposition to lower cholesterol levels. In the absence of associated findings in other examinations, the observation of decreased cholesterol is considered to be non-adverse.

There were minor increases in plasma triglyceride levels at 125 mg/kg/day (↑103% for males at week 1 and ↑150% for females at week 4).

There were no other changes in clinical biochemistry parameters considered to be related to treatment with the test item.

Some other inter-group variations occasionally achieved statistical significance but reflected changes which were present during pretest or did not show a dosage relationship and were considered not to be related to treatment with the test item. Higher levels of gamma-glutamyl transferase in males and plasma phosphorus in females at 125 mg/kg/day were within the 95th percentile of the historical control range. In addition, the concurrent control values for these parameters were at the low end of the historical control range.

Higher levels of plasma chloride in males at 125 mg/kg/day were within the 95th percentile of the historical control range.

Table 3: Intergroup comparison of clinical chemistry results

	Dose Level of bicyclopyrone (mg/kg/day)							
	Males				Females			
Parameter	**0**	**5**	**25**	**125**	**0**	**5**	**25**	**125**
Triglyceride (mmol/L)								
Pre-test	0.390 ± 0.110	0.525 ± 0.270	0.495 ± 0.084	0.410 ± 0.120	0.408 ± 0.186	0.465 ± 0.052	0.423 ± 0.154	0.428 ± 0.190
Week 1	0.313 ± 0.112	0.445 ± 0.187	0.473 ± 0.120	0.638* ± 0.186 (↑103%)	0.500 ± 0.227	0.428 ± 0.108	0.653± 0.367	0.483± 0.200
Week 4	0.330 ± 0.090	0.560 ± 0.225	0.478 ± 0.093	0.553 ± 0.114	0.320 ± 0.106	0.505 ± 0.097	0.428 ± 0.017	0.798** ± 0.161 (↑150%)
Week 8	0.500 ± 0.120	0.483 ± 0.145	0.475 ± 0.099	0.425± 0.132	0.308± 0.049	0.513** ± 0.051 (↑66%)	0.410± 0.059	0.430 ± 0.133
Week 13	0.475 ± 0.122	0.619 ± 0.370	0.535 ± 0.138	0.423 ± 0.117	0.418 ± 0.142	0.408 ± 0.084	0.428 ± 0.074	0.488 ± 0.074
Cholesterol (mmol/L)								
Pre-test	3.60 ±0.38	3.40 ±0.23	3.13 ±0.53	2.77* ±0.50 (↓23%)	3.57 ±0.74	3.25 ±0.49	3.28 ±0.64	3.76 ±0.56
Week 1	2.90 ±0.30	3.21 ±0.44	2.74 ±0.32	1.46** ±0.32 (↓50%)	3.18 ±0.59	2.77 ±0.42	2.58 ±0.54	1.80** ±0.40 (↓43%)
Week 4	2.90 ±0.21	2.99 ±0.24	2.59 ±0.44	1.55** ±0.41 (↓47%)	2.77 ±0.38	2.44 ±0.30	2.28 ±0.46	1.66** ±0.42 (↓40%)
Week 8	3.35 ±0.28	3.48 ±0.60	2.67 ±0.36	1.44** ±0.16 (↓57%)	3.02 ±0.78	2.69 ±0.31	2.39 ±0.60	1.94** ±0.27 (↓36%)
Week 13	3.45 ±0.27	3.48 ±0.83	2.80 ±0.43	1.30** ±0.36 (↓62%)	3.24 ±0.90	2.88 ±0.27	2.54 ±0.60	1.85** ±0.40 (↓43%)

Data were taken from pages 71-92 of the study report

Urinalysis: Ketones were apparently detected in the urine of all animals after treatment with bicyclopyrone during week 1, 6 and 13. Further laboratory investigations demonstrated that the tyrosine derived metabolite HPPA can cause a red-brown change in the urine dipstick used to

measure ketones. When using an automated method, this colour change was incorrectly identified as the presence of ketones in the urine.

Visual examination can clearly distinguish between the red-brown change caused by HPPA and the purple-violet change produced by acetoacetate. As an HPPD inhibitor, bicyclopyrone blocks the conversion of HPPA to homogentisate following HPPA production from tyrosine leading to elevated HPPA levels. The apparent detection of ketones in bicyclopyrone-treated animals in this study was due to cross-reactivity of HPPA with the dip-stick used to measure ketones.

No other changes were observed which were considered to be related to treatment with the test item.

Sacrifice and Pathology

Organ weights: Group mean absolute liver weights were higher in females treated at 125 mg/kg/day (↑38%). Adjusted liver weights were also statistically increased in this group (↑31%). In the absence of a similar finding in males or corroborative pathology and as there was no evidence of this finding following administration of bicyclopyrone for a period of 52 weeks, this finding was considered to be of no toxicological significance. See table 4.

Table 4: Absolute Liver Weights (g)

Dose Level of bicyclopyrone (mg/kg/day)							
Males				Females			
0	5	25	125	0	5	25	125
323 ± 24	321 ± 22	316 ± 16	351 ± 76	256 ± 40	269 ± 36	288 ± 26	353** ± 26 (↑38%)

Data were taken from pages 116 of the study report

There were no other changes in organ weights which were considered to be related to treatment with the test item. Statistically significantly decreased spleen weight in all bicyclopyrone-treated male groups, however, this was attributed to unusually high spleen weights in the concurrent control group.

Macroscopic findings: There were no macroscopic findings which were considered to be related to treatment with the test item. Any findings were considered incidental and commonly occur in dogs of this strain and age and under the experimental conditions used in this study.

Microscopic findings: There was an increased incidence and severity of inflammation/ fibrosis/apoptosis in the pancreas of male animals treated with the test item (minimal to slight). However, there was no coherent dose-response relationship and no evidence of a treatment-related effect in females. In addition, there was no evidence of this finding following administration of bicyclopyrone for a period of 52 weeks, indicating the presence of pancreatic findings in this study was unlikely to be related to treatment with bicyclopyrone. See table 5.

All other findings in this study occur commonly in laboratory dogs under the conditions of this study and the incidence, distribution and morphologic appearance did not indicate an association to treatment.

Table 5: Microscopic Findings

	Dose Level of bicyclopyrone (mg/kg/day)							
	Males				Females			
	0	**5**	**25**	**125**	**0**	**5**	**25**	**125**
No. of Animal	4	4	4	4	4	4	4	4
Pancreas – Inflammation/ fibrosis/ apoptosis	-	2 (Grades 1 and 2)*	2 (Both Grade 2)*	2 (Grades 1 and 2)*	1 (Grade 1)*	-	1 (Grade 1)*	-

Data were taken from pages 191 and 202 of the study report
*Grade1 = Minimal/very few/very small, Grade 2 = Slight/few/small, Grade 3 = Moderate/ moderate number/ moderate size, Grade 4 = Marked/ many/ large

INVESTIGATOR'S CONCLUSIONS: Treatment with bicyclopyrone at 5, 25 or 125 mg/kg/day resulted in an increase in liver weight in 125 mg/kg/day females only and a decrease in cholesterol levels in both sexes at 125 mg/kg/day. In the absence of any associated histopathological findings in the liver, these changes are considered to be non-adverse.

REVIEWER COMMENTS

All animals survived the scheduled treatment period. No clinical signs were observed that were considered to be related to treatment with the test item. There were no effects on food consumption and body weights which were considered to be related to treatment with the test item. Haematology parameters were unaffected by treatment with the test item. There were increased liver weights in females at the highest dose tested but there were no corroborating macroscopic or microscopic changes. There were no macroscopic findings in any organ which were considered to be related to treatment with the test item. Microscopically, an increased incidence of inflammation/fibrosis/apoptosis of the pancreas was observed in all treated male groups. These findings were mild in severity, and are likely to be transient as they were not observed in the chronic dog study.

Based upon the effects in this study, the NOAEL is 125 mg/kg bodyweight/day. The LOAEL was not observed.

This 90-day oral toxicity study in the dog is totally reliable (**acceptable/guideline**) and satisfies the guideline requirement for a 90-day oral toxicity study (OPPTS 870.3150; OECD 409) in dogs. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Jackson A, 2009)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar J. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) Date: 03/18/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) Date: 3/18/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: OECD 410 (1981): OPPTS 870.3200 (1998)

TEST MATERIAL (PURITY): NOA449280 (94.5 % w/w)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Sommer E.W., 2009. NOA449280: 28-day dermal toxicity study in Wistar rat. Harlan Laboratories (former RCC Ltd). Zelgliweg 1, 4452 Itingen , Switzerland. Laboratory Report No. B72101. 14 April 2009. Unpublished. (Syngenta File No. NOA449280_10999) MRID 47841978

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of this study.

EXECUTIVE SUMMARY

In a 28-day dermal toxicity study (MRID #47841978), groups of ten male and ten female Wistar rats were dermally exposed (using a semi-occlusive bandage) to bicyclopyrone (NOA449280) at dosages of 0 (control), 50, 250 or 1000 mg/kg/day for 6 hours a day, 5 days a week over a period of 28 days.

Clinical signs, food consumption and body weights were recorded periodically during acclimatization and treatment periods. During the final week of the treatment period, all animals from all groups underwent ophthalmoscopic examinations and were evaluated with a functional observation battery, which included measurement of grip strength and a quantitative measurement of locomotor activity. During the final week of treatment animals from the control and 1000 mg/kg/day groups underwent ophthalmic examination. At the end of the treatment period, blood samples were withdrawn for hematology and plasma chemistry analyses and urine samples were collected for urinalyses. All animals were subsequently killed; examined post mortem and a selection of organs/tissues were weighed. Histological examinations were performed on all animals from the control and high dose group. The eyes with optic nerve and harderian glands of low and mid dose group animals were also examined.

All animals survived the scheduled treatment period. No test item- related effects were recorded at the treated skin sites. There were no treatment-related effects on body weights or body weight gains. There were no treatment related adverse effects at 50 mg/kg/day. There were changes in clinical chemical parameters which were considered adaptive in nature. Increases in liver and kidney weights in males were not considered to be toxicologically significant in the absence of associated clinical pathology and histopathological findings. The corneal degeneration at 250 mg/kg/day in both sexes was not dose dependent (20% in males and 30% in females) and was of low severity. Both were grade 1 (minimal/small severity) and not considered adverse.

At 1000 mg/kg/day bicyclopyrone, there was a low incidence of vascular keratitis in males (20%) compared to 0% in the controls. The severity levels were grade 1- minimal/small and grade 3- moderate.

Based upon the effects of this study, the LOAEL is 1000 mg/kg/day, based upon ocular keratitis in males. The NOAEL is 250 mg/kg/day.

This study is classified as totally reliable (**acceptable/guideline)** and satisfies the guideline requirement for a 28-day dermal toxicity study (OPPTS 870.3200) in rats.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Item:	Bicyclopyrone (NOA449280)
Description:	brown beige powder
Lot/Batch number:	SEZ3AP006/MILLED
Purity:	94.5%
Stability of test compound:	Expiry date (retest date): 31 March 2011
Structures:	

Test Animals:	
Species:	Rat
Strain:	HanRcc: WIST (SPF)
Age/Weight at dosing:	Approximately 7 weeks at delivery, males: 187.0-244.70g, females: 134.2-173.8g
Housing:	Individually in Makrolon type-3 cages with wire mesh tops and standard softwood bedding ('Lignocel' Schill AG, 4132 Muttenz / Switzerland).
Acclimatisation period:	7 days
Diet:	Pelleted standard Kilba Nafag 3433 rodent maintenance diet *ad libitum*
Water:	Community tap water from Itingen was available *ad libitum* in water bottles.
Environmental conditions:	Temperature: 22 ± 3°C Humidity: 30-70%

Air changes: 10-15 air changes per hour
Photoperiod: 12 hours light /12 hours dark

Study Design and Methods:

In life dates: Start: 01 February 2008 End: 17 March 2008

Animal assignment: Animals were allocated to the study by computer-generated random algorithm.

Table 1: Study Design (animal allocation)

Allocation and dose levels (mg/kg/day)	Group 1 Control* 0	Group 2 50	Group 3 250	Group 4 1000	Group 10 Reserve animals**
Males	01-10	11-20	21-30	31-40	81
Females	41-50	51-60	61-70	71-80	82

Table was taken from page 17 of the study report
* Control animals were bandaged only, without application of test item.
** No reserve animals were required to be exchanged during the acclimatization period. These animals were removed from the study. Raw data collected during the acclimatization period on reserve animals are not reported but retained in the raw.

Route and duration of administration: Test substance administration was via the dermal route. The fur on the back of each rat was closely clipped as necessary (but at least once weekly) exposing an area of approximately 25 cm² (approximately 10% of the total body surface). For each animal, the test item was weighed out, directly placed onto the moistened skin with a spatula and covered with a bandage for 6 hours a day for a total of 5 days each week, over a 4 week period (total of 20 applications). After each 6 hour exposure period, the dressings were removed carefully and the treated area was gently rinsed with lukewarm tap water and the skin was dried with a disposable paper towel. The control animals were bandaged only, without application of test item.

Observations: Animals were observed twice daily for viability and mortality, and daily for skin reactions for erythema and edema before application and after removal of dressing. Detailed behavioural observations were recorded once weekly.

Bodyweight: Bodyweight was measured once weekly during the acclimatization and treatment period.

Food consumption: Food consumption was measured once weekly during the acclimatization and treatment period.

Ophthalmoscopic examination: For the conduct of ophthalmoscopic examinations, animals were randomized. Ophthalmoscopic examination was performed in all animals during acclimatization. During week 4, animals of groups 1 and 4 were examined after the treatment period on that day.

Neurobehavioural assessment:

Functional Observational Battery (FOB): During the last week of treatment and after the treatment period on that day, relevant parameters from a modified Irwin screen test were performed on all rats. These observations were based upon the procedures used for the detailed behavioral observations. Any abnormal findings were recorded and graded in severity.

Hind- and forelimb grip strength measurements were performed using a push-pull strain gauge (Mecmesin, AFG 25N).

Locomotor activity: Locomotor activity was measured quantitatively. Activity was measured with an AMS Föhr Medical Instruments GmbH (FMI) and DeMeTec GmbH. Activity of the animals was recorded for 10-minute intervals over a period of 60 minutes. These data and the total activity over 60 minutes are reported.

Haematology and clinical chemistry: Blood samples were drawn from the retro-orbital plexus from all animals, in a random order, under light isoflurane anesthesia. The animals were fasted for approximately 18 hours before blood sampling but allowed access to water ad libitum. The samples were collected early in the working day to reduce biological variation caused by circadian rhythms. Urine was collected from all animals during the 18 hours fasting period into a specimen vial, using a metabolism cage.

The following haematology parameters were determined:

Erythrocyte count
Leukocyte count, total
Hematocrit
Mean corpuscular volume
Red cell volume distribution width
Mean corpuscular hemoglobin
Mean corpuscular hemoglobin concentration
Hemoglobin concentration distribution width
Reticulocyte count
Reticulocyte maturity index (low, medium, high fluorescence)
Coagulation**:** Prothrombin time = Thromboplastin time /Activated partial thromboplastin time
Hemoglobin
Differential leukocyte count:
Neutrophils
Eosinophils
Basophils
Lymphocytes
Monocytes
Large unstained cells
Platelet count
Haemoglobin derivatives: Methemoglobin / Heinz bodies

Clinical chemistry: The following parameters were determined:

Glucose
Urea
Creatinine
Bilirubin, total
Cholesterol, total
Triglycerides
Aspartate aminotransferase
Alanine aminotransferase
Lactate dehydrogenase
Glutamate dehydrogenase
Alkaline phosphatase
Gamma-glutamyl-transferase
Creatine kinase
Sodium
Potassium
Chloride
Calcium
Phosphorus, inorganic
Protein, total
Albumin
Globulin
Albumin/Globulin ratio

Urinalysis: The following urine parameters were determined:

Urine volume (18 hour)
Specific gravity (relative density)
pH value
Nitrate
Protein
Glucose
Ketones
Colour
Appearance
Urobilinogen
Bilirubin
Erythrocytes
Leukocytes

Investigations *post mortem:*

All animals were anesthetized by intraperitoneal injection of pentobarbitone and killed by exsanguination.

Samples of the following tissues and organs were collected from all animals at necropsy and, unless otherwise indicated, fixed in neutral phosphate buffered 4% formaldehyde solution. Additional tissues (such as ear tattoo) were retained in accordance with standard operating procedures but were not processed or examined further.

Histopathology: Slides of all organs and tissues listed in boldface type collected at terminal sacrifice from the animals of the control and high dose groups were examined by the study pathologist. In addition, all gross lesions noted at necropsy were examined histologically. Due to potential test item-related effects, the eyes with optic nerve and Harderian glands of low and mid dose group animals were examined.

Adrenal glands
Aorta
Bone (sternum, femur including joint)
Bone marrow (femur)
Brain – including section of medulla/pons, cerebral and cerebellar cortex
Cecum
Colon
Duodenum
Epididymides (fixed in Bouin's solution)
Esophagus
Eyes w/optic nerve (fixed in Davidson's solution)
Harderian gland (fixed in Davidson's solution)
Heart including auricles
Ileum, with Peyer's patches
Jejunum with Peyer's patches
Kidneys
Larynx
Lacrimal gland, exorbital
Liver
Lungs, filled w/formalin at necropsy
Lymph nodes - mesenteric and mandibular
Mammary gland area
Nasal cavity
Ovaries
Pancreas Pharynx
Pituitary gland
Prostate gland incl. coagulating glands
Rectum
Salivary glands - mandibular, sublingual
Sciatic nerve
Seminal vesicles
Skeletal muscle
Skin (treated and untreated)
Spinal cord - cervical, mid thoracic, lumbar
Spleen
Stomach
Testes (fixed in Bouin's solution)
Thymus
Thyroid (incl. parathyroid gland, if possible)
Tongue
Trachea
Urinary bladder (filled w/formalin at necropsy)
Uterus
Vagina
Gross lesions

Organ weights: The following organ weights were recorded on the scheduled dates of necropsy and their organ to terminal body weight ratios determined:

Adrenal glands	Kidneys	Testes
Brain	Liver	Thymus
Epididymides	Ovaries	Uterus
Heart	Spleen	

Microscopic examination: All organ and tissue samples to be examined by the study pathologist were processed, embedded and cut at an approximate thickness of 2 - 4 micrometers and stained with hematoxylin and eosin.

Statistics: Statistical analysis was performed by Datametrix AG, Technoparkstrasse 1, 8005 Zürich / Switzerland. The following statistical approaches were used in this study:

• All analyses were two-tailed for significance levels of 5% and 1%.

• All means are presented with standard deviations.

• Where the variances were clearly heterogeneous (male absolute neutrophils only), a log transformation was used to stabilize the variances.

• For continuous data: body weights, cumulative body weight gain, food consumption, clinical pathology values (hematology, clinical chemistry, and urinalysis) and absolute organ weights were analyzed initially by a one-way analysis of variance (ANOVA).

• Organ weights were also analyzed by analysis of covariance (ANCOVA) on final body weight. This statistical analysis provided an Adjusted Organ Weight value, which is displayed in the results table in this report along with flags for statistical significance.

• Summary statistics of organ to body weight ratios are presented but these were not analysed statistically.

• For all of the parameters evaluated initially by ANOVA or ANCOVA, Dunnett's test was used to compare the control and treated groups, based on the error mean square in the ANOVA or ANCOVA.

• For discontinuous or descriptive data (e.g. present/absent): Parameters that yield discontinuous or descriptive data were analyzed by Fisher's Exact Test.

• Ophthalmoscopy, macropathology and micropathology incidence data were analysed using Fisher's Exact Test.

Individual values were rounded before printing. All derived values that appear in the report tables represent the rounded results of calculations that are based on the exact (non-rounded) raw data values. Statistical analyses also were carried out on the exact raw data values.

RESULTS AND DISCUSSION

Mortality: There were no mortalities.

Clinical observations: No test item-related effects were recorded at the treated skin sites. No treatment-related clinical signs were observed.

Food consumption: There were no treatment-related effects on food consumption.

Bodyweights: No statistically significant treatment-related effects were noted on body weight (see table 2) or body weight gain.

Table 2: Mean intergroup comparison of bodyweights (g)

	bicyclopyrone (mg/kg/day)							
	Males				Females			
days	0	50	250	1000	0	50	250	1000
1	259.25 ± 12.08	260.59 ± 11.57	261.04 ± 15.07	263.28 ± 17.43	169.68 ± 12.55	172.49 ± 12.80	179.06 ± 8.98	173.32 ± 10.96
8	282.20 ± 13.95	281.53 ± 13.19	282.57 ± 17.49	283.74 ± 19.47	187.47 ± 9.87	190.14 ± 14.22	192.96 ± 12.10	186.99 ± 12.60
15	305.80 ± 16.68	301.41 ± 15.93	302.41 ± 22.03	303.13 ± 20.48	205.51 ± 14.30	206.48 ± 19.66	210.49 ± 7.93	201.19 ± 12.83
22	327.12 ± 18.71	320.74 ± 19.10	319.37 ± 23.26	322.84 ± 22.33	217.63 ± 16.01	221.97 ± 23.38	224.70 ± 11.00	212.08 ± 15.34
28	333.48 ± 18.40	327.51 ± 20.68	325.70 ± 25.60	331.69 ± 24.58	220.95 ± 13.76	223.00 ± 21.82	224.37 ± 11.35	215.69 ± 14.02

Data were taken from pages 61-62

Ophthalmoscopic examination: No treatment-related changes to the eyes were observed.

Neurobehavioral findings:

Grip strength: No effects on grip strength were detected.

Locomotor activity: No effects on locomotor activity were detected.

Haematology: No changes were noted in any of the haematology parameters examined that were attributable to treatment.

Any differences between the values in test item-treated and control animals, although on occasions achieving statistical significance, were minor, not related to dosage, not consistent across the sexes or within the normal background ranges recorded for rats of this strain and age and were considered to be incidental to treatment.

Clinical chemistry: Alkaline phosphatase (ALP) activity was lower in all test item-treated male groups ($p < 0.01$), when compared to the values in the control group (↓35-42%).

Creatinine levels in all test item-treated male groups were lower than that of the concurrent control group (↓12-16%). However, the group mean value of the control group was at the upper limit of the normal background range recorded for rats of this strain and age, and levels within all

treatment groups were within the historical control range. These differences were considered to be incidental to treatment.

All other differences between the values in test item-treated and control animals, although on occasions achieving statistical significance, were minor, not related to dosage, not consistent across the sexes and/or within the normal background ranges recorded for rats of this strain and age and were considered not to be related to treatment.

Table 3: Clinical Chemical Changes

	bicyclopyrone (mg/kg/day)							
	Males				Females			
	0	50	250	1000	0	50	250	1000
Alkaline Phosphatase (U/L)	111.2 ± 34.5	72.6** ± 26.1 (↓35%)	64.7** ± 22.0 (↓42%)	68.6** ± 9.7 (↓38%)	52.0 ± 19.9	50.1 ± 15.7	43.0 ± 10.5	45.6 ± 13.1
Creatinine (µmol/L)	25.5 ± 1.9	22.5** ± 2.1 (↓12%)	22.2** ± 1.0 (↓13%)	21.3** ± 2.7 (↓16%)	24.3 ± 2.0	23.2 ± 3.6	24.6 ± 3.0	22.4 ± 4.1

Data were taken from pages 85-88.
*Statistically significant difference from the control group mean at the 5% level (Student's t-test, two-sided).
** Statistically significant difference from the control group mean at the 1% level (Student's t-test, two-sided).

Urinalysis: It appeared that ketone levels in the urine were increased in a dosage-related manner in all test item-treated male and female groups. Further laboratory investigations demonstrated that the tyrosine derived metabolite HPPA can cause a red-brown change in the urine dipstick used to measure ketones. When using an automated method, this colour change was incorrectly identified as the presence of ketones in the urine.

Visual examination can clearly distinguish between the red-brown change caused by HPPA and the purple-violet change produced by acetoacetate. As an HPPD inhibitor, bicyclopyrone blocks the conversion of HPPA to homogentisate following HPPA production from tyrosine leading to elevated HPPA levels. The apparent detection of ketones in bicyclopyrone-treated animals in this study was due to cross-reactivity of HPPA with the dip-stick used to measure ketones.

Any other differences between the values in test item-treated and control animals, although on occasions achieving statistical significance, were considered to be unrelated to treatment as they were minor, not related to dosage, not consistent across the sexes or within the normal background ranges recorded for rats of this strain and age.

Sacrifice and pathology:

Organ weights: Dose-related higher absolute liver weights were observed in all male dose groups with statistical significance at 1000 mg/kg/day and there were statistically significant increases in the adjusted liver weights in all treatment groups (see table 4). The observed increases were less than 20% above concurrent control values and represent a shallow dose response across the range of dose levels tested. Statistically significantly increased adjusted kidney weights were recorded in male rats treated either with 250 mg/kg/day or 1000 mg/kg/day. These organ weight increases are unlikely to be toxicologically significant, in the absence of associated clinical pathology or histopathological findings.

There were no other treatment-related effects on organ weights. Increased adrenals weight was noted in females treated with 250 mg/kg/day. However, in absence of any difference at 1000 mg/kg/day this was considered to be spurious.

Table 4: Absolute and Adjusted Liver and Kidney Weights

	bicyclopyrone (mg/kg/day)							
	Males				Females			
	0	50	250	1000	0	50	250	1000
Liver (Abs.)	9.27 ± 0.9	9.98 ± 1.21	10.19 ± 1.20	10.73* ± 1.25 (↑16%)	6.56 ± 0.91	6.83 ± 0.96	7.32 ± 0.71	6.92 ± 0.61
Liver (Adj.)	9.083	10.093* (↑11%)	10.298** (↑13%)	10.694** (↑18%)	6.521	6.764	7.198	7.141
Kidney (Abs.)	1.97 ± 0.15	2.05 ± 0.22	2.10 ± 0.17	2.16* ± 0.16 (↑10%)	1.48 ± 0.13	1.44 ± 0.14	1.57 ± 0.12	1.46 ± 0.15
Kidney (Adj.)	1.945	2.062	2.114* (↑9%)	2.159* (↑11%)	1.476	1.434	1.548	1.494

Data were taken from pages 93-98
*Significant at 5%
**Significant at 1%

Macroscopic findings: All lesions recorded during the macroscopic observation were deemed to be unrelated to treatment and were within the range of background alterations that may be recorded in this type of study, in rats of this strain and age.

Microscopic findings: A minimal degree of degeneration of the corneal epithelium was noted in the eyes of 2 males and 3 females treated with 250 mg/kg/day. In 2 males treated with 1000 mg/kg/day minimal to moderate degrees of keratitis were recorded.

Other lesions recorded during the microscopic observation were within the range of background alterations that may be recorded in this type of study, and in rats of this strain and age.

Table 5: Microscopic findings (Animals affected)

	bicyclopyrone (mg/kg/day)							
	Males				Females			
	0	50	250	1000	0	50	250	1000
Keratitis	0	0	0	2 (Severity levels 1 and 3*)	0	0	0	0
Corneal Degeneration	0	0	2 (Severity level 1*)	0	0	0	3 (Severity level 1)	0

Data were taken from pages 144-163
*Grade1 = Minimal/very few/very small, Grade 2 = Slight/few/small, Grade 3 = Moderate/ moderate number/ moderate size, Grade 4 = Marked/ many/ large

CONCLUSION: The only toxicologically significant findings following dermal administration of bicyclopyrone to Wistar rats at doses of 50, 250 or 1000 mg/kg/day for 6 hours a day for 5 days each week over a period of 28 days were eye lesions (keratitis or degeneration of corneal epithelium) in a low number of animals at 250 and 1000 mg/kg/day.

Based upon the effects of this study, the LOAEL is 250 mg/kg/day, based upon eye lesions (keratitis or degeneration of corneal epithelium) in males. The NOAEL is 50 mg/kg/day.

REVIEWER COMMENTS:

In a 28-day dermal toxicity study (MRID #47841978), groups of ten male and ten female Wistar rats were dermally exposed (using a semi-occlusive bandage) to bicyclopyrone (NOA449280) at dosages of 0 (control), 50, 250 or 1000 mg/kg/day for 6 hours a day, 5 days a week over a period of 28 days.

The incidence of the ocular keratitis is low in males at the high dose. One of the animals is grade 1 for severity while the other is grade 3 suggesting some degree of adversity. These effects are consistent the toxicity profile of bicyclopyrone in rats, and though it is conservative, the LOAEL will be set at 1000 mg/kg/day.

Based upon the effects of this study, the LOAEL is 1000 mg/kg/day, based upon ocular keratitis in males. The NOAEL is 250 mg/kg/day.

This study is classified as totally reliable (**acceptable/guideline)** and satisfies the guideline requirement for a 28-day dermal toxicity study (OPPTS 870.3200) in rats. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Sommer E, 2009)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Prenatal Developmental Study (rat) OECD 414 (2004): EC 2004/73 B31, (2004): OPPTS 870.3700 (1998): JMAFF 12NohSan No. 8147 (2000)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Gerspach R, 2011. NOA449280: Prenatal developmental toxicity study in the Han Wistar rat. Harlan Laboratories Ltd., Füllinsdorf, Switzerland. Laboratory Report No. C41887, 24 November 2011. Unpublished. (Syngenta File No. NOA449280_11150) MRID 47841993

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, UK.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a developmental toxicity study in rats (MRID #47841993), Bicyclopyrone (NOA449280, 94.5%) was administered by oral gavage to 24 mated female RccHan: WIST(SPF) in 0.5% carboxymethyl cellulose (CMC) in purified water at dose levels of 0, 100, 500 and 1000 mg/kg bw/day on days 6 to 20 of gestation. All females were killed on day 21 post coitum and the foetuses were removed by Caesarean section. Examination of dams and foetuses was performed in accordance with international recommendations. All animals survived until scheduled necropsy.

Maternal effects are as follows:

No treatment-related clinical signs were noted. Decreases in body weight gains and food consumption were observed at all dose levels but were not considered adverse due to lack of effects on absolute maternal body weights. There was also no effect on any of the evaluated reproduction parameters.

Based upon the effects in this study, the maternal NOAEL is 1000 mg/kg bw/day. The maternal LOAEL was not observed.

Foetal effects are as follows:

Effects on the foetuses were found at all tested dose levels. Foetal weights were decreased at 500 and 1000 mg/kg/day (↓6-15%). Skeletal evaluation revealed an increased incidence of full or rudimentary supernumerary ribs, accompanied by an increased incidence of pelvic girdle malpositioned (caudal), and an increased incidence of long costal cartilage 11, at 100, 500 and/or 1000 mg/kg/day.

Increased incidences of non-ossified vertebral bodies occurred at all dose levels. In addition, statistically significant increased incidences of a number of variations of bone or cartilage structures and of delays in ossification occurred at the 500 and 1000 mg/kg/day dose level.

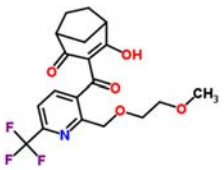
Based upon the effects in this study, the developmental LOAEL is 100 mg/kg bw/day based upon various skeletal variations. The developmental NOAEL was not observed.

This study is classified as totally reliable (**acceptable/guideline)** and satisfies the registrants' need (OPPTS 870.3700a; OECD 414) for a developmental toxicity study in rats.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS
Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Technical, brown beige powder
Lot/Batch number:	SEZ3AP006/Milled
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Stability confirmed (stored at a temperature < 30°C; light protected, dry)
Structure:	



Vehicle and/or positive control: The test substance was administered in 0.5% w/v aqueous carboxymethylcellulose (CMC) high viscosity.

Test Animals:

Species	Rat
Strain	RccHan: WIST(SPF)
Age/weight at dosing	11 weeks / 180-220 g
Source	Harlan Laboratories Ltd., Laboratory Animal Services, Wölferstrasse 4, 4414 Füllinsdorf, Switzerland
Housing	Individually in Makrolon type-3 cages with wire mesh tops
Acclimatisation period	At least 7 days
Diet	Pelleted standard Kliba-Nafag 3433 rat/mouse maintenance diet (Provimi Kliba SA, 4303 Kaiseraugst, Switzerland) *ad libitum*
Water	Tap water *ad libitum*
Environmental conditions	Temperature: 22±3°C Humidity: 30-70% Air changes: 10-15 per hour Photoperiod: 12 hour fluorescent light/12 hour dark

Study Design and Methods:

In-life dates: Start: 19 May 2009 End: 12 June 2009

Mating procedure: After acclimatisation females were housed with sexually mature males (1:1) in special mating cages i.e. with synchronized timing to initiate the nightly mating period, until evidence of copulation was observed. This system reduced the variation in the copulation times of the different females. The females were removed and housed individually if a copulation plug was observed, and / or the daily vaginal smear was sperm positive. The day of mating was designated day 0 post coitum. (Male rats of the same source and strain were used only for mating and were not considered part of the test system. The fertility of these males had been proven and was continuously monitored).

Animal assignment: Animals were randomly assigned to test groups, using a computer-generated random algorithm, as shown in the following table:

Table 1: Animal numbers and treatment groups

	Dose level of bicyclopyrone (mg/kg bw/day)			
Females	**0 (control)**	**100**	**500**	**1000**
	1-24	25-48	49-72	73-96

Table was taken from page 18 of the study report

Dose selection rationale: The dose levels were selected based on a previous dose range-finding toxicity study in Han Wistar rats, Harlan Laboratories Study B50512 (MRID #47841992), using dose levels of 0, 100, 500 and 1000 mg/kg/day (See Appendix).

Route and duration of administration: Bicyclopyrone was administered by gavage on days 6 to 20 of gestation to groups of time mated female rats (24/dose).

Dosage preparation and analysis: The dose suspensions were prepared weekly, based on results of stability testing of suspension ranging from 1 to 120 mg/mL using the test item as supplied by the Sponsor. Bicyclopyrone was weighed into a glass beaker on a tared precision balance and the vehicle was added (w/v). The preparation was homogenised for about 20 minutes before subdividing. The preparation was subdivided into separate aliquots for dosing each day. Homogeneity of the test item in the vehicle was maintained during the daily administration period using a magnetic stirrer. The dosing formulations were stored at 2-8ºC.

On the first treatment day, samples from the control group as well as three samples (top, middle and bottom) of about 2 g of each concentration were taken prior to dosing for analysis of concentration and homogeneity. Samples of about 2 g of each concentration were taken from the middle only to confirm stability (7 days). During the last week of the treatment, samples were taken from the middle to confirm concentration. The aliquots for analysis of dose formulations were frozen (-20±5°C) until analysis. The samples were analysed by HPLC.

Concentration analysis results: The test item concentration in all samples was 91.1% to 108.6% of the nominal concentration.
Homogeneity results: Homogenous distribution was demonstrated because single results found did not deviate more than 6.5% (<15%) from the corresponding mean.
Stability results: The dosing formulations were considered to be stable for at least 7 days under storage conditions.

The analytical data indicate that the test substance was homogeneous and stable in the suspensions and that the variation between the nominal and actual dosages to the animals was acceptable.

Dosage administration and duration: A standard dose volume of 10 mL/kg body weight/day with a daily adjustment to the actual body weight was used. Control animals were dosed with the vehicle alone (0.5% w/v aqueous carboxymethylcellulose, CMC, high viscosity). All rats were dosed from day 6 to day 20 post coitum (the day prior to Caesarean section).

Observations:
Maternal observations: Animals were examined/observed for signs of toxicity and mortality twice daily. Clinical signs were assessed at least twice daily for signs of reaction to the treatment and / or symptoms of ill health. Body weights were recorded daily from day 0 until day 21 post coitum. Food consumption was recorded at 3 day intervals: days 0 - 3, 3-6, 6-9, 9-12, 12-15, 15-18 and 18-21 post coitum. At the scheduled necropsy on day 21 post coitum, females were sacrificed by CO_2 asphyxiation and the foetuses removed by Caesarean section. Post mortem examination, including gross macroscopic examination of all internal organs with emphasis on the uterus, uterine contents, position of foetuses in the uterus and the number of corpora lutea was performed and the data recorded. The uteri (and contents) of all females with live foetuses were weighed during necropsy on day 21 post coitum to enable the calculation of the corrected body weight gain.

Foetal observations: Foetuses were removed from the uterus, sexed, weighed individually, examined for gross external abnormalities, killed by a subcutaneous injection of sodium pentobarbital and allocated to one of the following procedures:

1. At least one half of the foetuses from each litter was fixed in Bouin's fixative and examined by a combination of serial sections of the head and microdissection of the thorax and abdomen. This included detailed examination of the major blood vessels and sectioning of the heart and kidneys. After examination, the tissues were preserved in a solution of glycerin/ethanol. Descriptions of any abnormalities and variations were recorded.

2. The remaining foetuses were eviscerated and with the exception of over the paws, the skin was removed and discarded. Carcasses were processed through solutions of ethanol, glacial acetic acid with Alcian blue (for cartilage staining), potassium hydroxide with Alizarin red S (for clearing and staining ossified bone) and aqueous glycerin for preservation and storage. The skeletons were examined and all abnormalities and variations were recorded. The specimens were preserved individually.

If no implantation sites were evident, the uterus was placed in an aqueous solution of ammonium sulphide to accentuate possible haemorrhagic areas of implantation sites.

Foetuses with abnormalities were photographed.

Indices: The following indices were calculated from caesarean section records of animals in the study: Pre and post-implantation losses, embryonic and foetal deaths, live and dead foetuses, abnormal foetuses, foetal sex ratios and foetal body weights. For reproduction data, group mean values were calculated both on a litter basis and on a percentage per group basis. Mean foetal weights were calculated from the individual weights on a per litter basis.

Historical control data: Historical control data were provided for all reproductive and foetal findings observed in the current study. These historic control data were taken from 10 studies in the same strain of rat, evaluated by similar foetal examination criteria, in this laboratory in 2007 and 2008.

Statistical analyses: The following statistical methods were used to analyse maternal, reproduction and skeletal examination data:
• Means and standard deviations of various data were calculated.
• All statistical tests were two-sided.
• Statistical significance between groups was evaluated by Analysis of Variance (ANOVA). In the case where variances were non-homogeneous, appropriate transformations were applied (e.g. log, square root, or double arcsine) to stabilise the variances before the ANOVA. The Dunnett many-to-one t-test was then used to compare each group to control based on the error mean square in the ANOVA.
• Fisher's Exact-test was applied if the variables could be dichotomised without loss of information.
• For statistical tests on foetal data, comparisons were made between groups for number of foetuses affected and number of litters affected, for completeness. The litter was considered the proper unit of measurement for overall study evaluation.

RESULTS

Maternal toxicity:
Mortality and clinical signs: All animals survived until scheduled necropsy. No test item-related clinical signs were noted.

Body weight: There was no effect on absolute body weights (see table 2). Dose-related decreases in maternal body weight gain (see table 3) were observed at all dose levels beginning immediately after initiation of treatment. These differences, though transient at 100 mg/kg/day, persisted throughout treatment at 500 and 1000 mg/kg/day. Mean maternal body weight gain (corrected for gravid uterus weight) was also decreased >10% at 500 and 1000 mg/kg/day.

Table 2: Intergroup comparison of absolute maternal body weights (g) – selected times

	Dose level of bicyclopyrone (mg/kg bw/day)			
day	**0 (control)**	**100**	**500**	**1000**
7	226 ± 9.9	230 ± 8.6	225 ± 7.1	225 ± 10.1
14	258 ± 11.5	263 ± 10.0	255 ± 9.3	253 ± 11.9
21	337 ± 19.0	340 ± 16.5	331 ± 16.7	327 ± 23.7

Data were taken from pages 35 and 36 of the study report
* / ** Dunnett-Test based on pooled variance significant at 5% (*) or 1% (**) level

Table 3: Intergroup comparison of maternal body weight gain (g) – selected times

	Dose level of bicyclopyrone (mg/kg bw/day)			
day	**0 (control)**	**100**	**500**	**1000**
6-7	4.23 ± 2.23	0.86** ± 3.00	-0.01** ± 2.28	0.12** ± 3.01
6-14	36.9 ± 4.26	34.1 ± 4.22	29.8* ± 5.45 (↓19%)	27.9** ± 6.85 (↓24%)
6-20	103.8 ± 11.8	97.2 ± 12.1	91.1** ± 13.0 (↓12%)	85.6** ± 17.4 (↓18%)
6-21	115.5 ± 13.9	110.8 ± 14.1	105.9 ± 15.1	102.0* ± 20.0 (↓12%)

Data were taken from pages 37 and 38 of the study report
* / ** Dunnett-Test based on pooled variance significant at 5% (*) or 1% (**) level

Food consumption: At 500 and 1000 mg/kg/day statistically significantly decreased food consumption was observed (↓7-14%).

Table 4: Intergroup comparison of maternal food consumption (g/rat/day) – selected times

day	Dose level of bicyclopyrone (mg/kg bw/day)			
	0 (control)	**100**	**500**	**1000**
3-6	18.9 ± 2.2	20.3 ± 2.0	20.1 ± 2.0	19.5 ± 2.2
6-9	22.5 ± 2.1	22.2 ± 1.9	20.9* ± 2.0 (↓7%)	19.4** ± 2.4 (↓14%)
12-15	23.4 ± 1.9	24.3 ± 2.2	22.7 ± 2.2	21.7* ± 1.9 (↓7%)
18-21	24.8 ± 1.8	25.2 ± 1.8	25.1 ± 2.0	23.9 ± 1.8

Data was taken from page 34 of the study report
* / ** Dunnett-Test based on pooled variance significant at 5% (*) or 1% (**) level

Reproduction data: There was no effect on any of the evaluated reproduction parameters (post-implantation loss, embryonic or foetal resorptions, dead or live foetuses) in any dose group.

Sacrifice and pathology:
Gross pathology: No findings were noted in any dam at scheduled necropsy. One dam of the high dose group (no. 84) had a bilateral dilated kidney pelvis, which was considered to be incidental, due to its isolated occurrence.

Caesarean section data: Data are summarised in the table 5 below:

Table 5: Caesarean section observations for all pregnant females

Observation	Dose level of bicyclopyrone (mg/kg bw/day)			
	0 (control)	100	500	1000
Number of dams	24	24	24	24
Corpora lutea (number)	330	329	337	317
(mean) *	13.8 ± 1.8	13.7 ± 2.2	14.0 ± 1.9	13.2 ± 2.3
Pre-implantation loss (number)	17	28	32	10
(% corpora lutea) +	5.2	8.5	9.5	3.2
(mean)	0.7 ± 0.7	1.2 ± 1.5	1.3 ± 1.8	0.4 ± 0.6
(no dams affected)	14	13	15	9
Implantation sites (number)	313	301	305	307
(% corpora lutea)	94.8	91.5	90.5	96.8
(mean) *	13.0 ± 1.8	12.5 ± 2.4	12.7 ± 2.2	12.8 ± 2.2
Post-implantation loss (number)	14	25	17	19
(% implantation sites) +	4.5	8.3	5.6	6.2
(mean)	0.6 ± 0.9	1.0 ± 1.0	0.7 ± 1.3	0.8 ± 1.0
(no dams affected)	9	15	9	13
Embryonic foetal deaths (total)	14	25	17	19
Embryonic resorptions (number)	14	24	16	18
(% implantation sites) +	4.5	8.0	5.2	5.9
(mean) +	0.6 ± 0.9	1.0 ± 1.0	0.7 ± 1.3	0.8 ± 1.0
Foetal resorptions (number)	0	1	1	1
(% implantation sites) +		0.3	0.3	0.3
(mean) +		0.0 ± 0.2	0.0 ± 0.2	0.0 ± 0.2
(no of dams affected)		1	1	1
Total number of foetuses	299	276	288	288
(% implantation sites)	95.5	91.7	94.4	93.8
(mean) *	12.5 ± 2.1	11.5 ± 2.6	12.0 ± 2.6	12.0 ± 2.8
Live foetuses	299	276	288	288
Dead foetuses	0	0	0	0
Number of male foetuses	161	131	147	147
(% of foetuses) +	53.8	47.5	51.0	51.0
Mean wt (g) of live foetuses (litter basis) (total) *	4.9 ± 0.2	4.8 ± 0.3	4.6** ± 0.3 (↓6%)	4.2** ± 0.3 (↓15%)
Mean wt (g) of live foetuses (litter basis) (male) *	5.0 ± 0.2	4.9 ± 0.3	4.7** ± 0.3 (↓6%)	4.3** ± 0.4 (↓14%)
Mean wt (g) of live foetuses (litter basis) (female) *	4.8 ± 0.2	4.7 ± 0.3	4.4** ± 0.3 (↓8%)	4.1** ± 0.3 (↓15%)

Data were taken from pages 43-44 of the study report

*/** : Dunnett-Test based on pooled variance significant at level 5% (*) or 1% (**)

+/++ : Dunnett-Test after double arcsine transformation of the proportion, based on pooled variance significant at level 5% (+) or 1% (++)

Developmental toxicity: Effects on the foetuses were found at all tested dose levels. Foetal weights were decreased at 500 and 1000 mg/kg/day (↓6-15%).

External examinations: There were no treatment related external effects.

Visceral examinations: Statistically significant increases in the litter incidences of supernumerary median cleft of the liver were observed at 1000 mg/kg/day (11 litters) and 500 mg/kg/day (12 litters). These incidences were outside the historical control range, and though apparently related to treatment, were considered not to be adverse.

Skeletal examinations: Data for skeletal variations are presented in table 5. Skeletal evaluation revealed increased incidence of full or rudimentary supernumerary ribs at 1000 mg/kg/day, 500 mg/kg/day, and/or 100 mg/kg/day; accompanied by an increased incidence of pelvic girdle malpositioned (caudal), and an increased incidence of long costal cartilage 11 at 1000 mg/kg/day, 500 mg/kg/day, and at 100 mg/kg/day. Additionally, a small but statistically significant increased incidence of incompletely ossified sternebrae 2 was observed from 100 mg/kg/day and above, but no other sternbrae were incompletely ossified.

Increased incidence of non-ossified vertebral bodies occurred at all dose levels. In addition, statistically significant increased incidences of a number of variations of bone or cartilage structures and of delays in ossification occurred at the 500 and 1000 mg/kg/day dose levels.

Table 5: Intergroup comparison of selected foetal skeletal findings: number of foetuses affected

Finding	Dose level of bicyclopyrone (mg/kg bw/day)			
	0 (control)	100	500	1000
Number of foetuses (litters) examined	144 (24)	132 (24)	136 (24)	139 (24)
Pelvic girdle malpositioned (caudal)	5 (4)	19** (12)	54** (20)	61** (18)
Sternebrae offset ossification sites	0	3 (3)	3 (3)	11** (8)
Sternebrae bipartite ossification	0	2 (2)	1 (1)	6* (4)
Costal cartilages asymmetrically aligned at the sternum	3 (3)	8 (7)	8 (6)	14** (9)
Non ossified cervical vertebral Body 1	18 (0)	11 (0)	5** (1)	12 (0)
Non ossified cervical vertebral Body 2	10	43**	49**	87**
Non ossified cervical vertebral Body 3	5	35**	39**	87**
Non ossified cervical vertebral Body 4	6	25**	25**	71**
Non ossified cervical vertebral Body 5	0	20**	20**	56**
Non ossified cervical vertebral Body 6	1	9**	10**	48**
Non ossified cervical vertebral Body 7	0	2	3	20**
Non ossified caudal vertebrae, some	1	3	4	35**
Sternum incompletely ossified Sternebra 1	0	0	1	2
Sternum incompletely ossified Sternebra 2	0	9**	5*	12**
Sternum incompletely ossified Sternebra 3	0	1	0	4
Sternum incompletely ossified Sternebra 4	0	2	0	2
Sternum incompletely ossified Sternebra 5	13	17	11	28*
Sternum incompletely ossified Sternebra 6	0	3	5*	17**
Supernumerary, one rib, left	2	4	22**	27**
Supernumerary, one rudimentary rib, left	15	58**	77**	79**
Supernumerary, one rib, right	1	4	19**	27**
Supernumerary, one rudimentary rib, right	19	53**	68**	73**
Long costal cartilage 11, left[1]	2	10*	12**	17**
Long costal cartilage 11, right[1]	2	11**	15**	14**
Supernumerary costal cartilage, left[1]	1	2	12**	13**
Supernumerary costal cartilage, right[1] (per-litter)	1	4	9*	13**

Data were taken from pages 56-63 of the study report
* / ** Fisher's Exact Test significant at 5% (*) or 1% (**) level
[1] per litter (not per fetus)

INVESTIGATOR'S CONCLUSION:

In this developmental toxicity study in the Han Wistar rat, 100 mg/kg bw/day was considered the LOEL for both maternal and foetal effects.

REVIEWER'S COMMENTS:

Based upon the effects in this study, the maternal NOAEL is 1000 mg/kg bw/day. The maternal LOAEL was not observed.

Based upon the effects in this study, the developmental LOAEL is 100 mg/kg bw/day based upon various skeletal variations. The developmental NOAEL was not observed.

APVMA notes that in this study, clear statistically significant decreases in bodyweight gains were noted from GD 7–11 (-80% on GD7, -37% on GD8, etc…see p37 of study report) when compared with control. APVMA/OCS and PMRA both believe that the decrease in maternal bodyweight gains are adverse and would thus set the maternal LOAEL at the lowest dose tested (100 mg/kg/day). As a matter of policy, EPA does not consider decreases in body weight gains to be adverse.

This study is classified as totally reliable (**acceptable/guideline)** and satisfies the requirement (OPPTS 870.3700a; OECD 414) for a developmental toxicity study in rats. EPA, PMRA (Canada), APVMA/OCS (Australia) agree on the classification for this study, but not the regulatory decision.

(Gerspach R, 2011)

Appendix

STUDY TYPE: Prenatal Developmental Study – range-finding (rat) OECD 414 (2001): OPPTS 870.3700 (1998): JMAFF 12 NohSan No. 8147

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Gerspach R, 2011. NOA449280: Dose range finding prenatal developmental toxicity study in the Han Wistar rat. Harlan Laboratories Ltd., Füllinsdorf, Switzerland. Laboratory Report No. B50512, 29 November 2011. Unpublished. (Syngenta File No NOA449280_11149) MRID 47841992

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, UK.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

This was a dose-finding study based on the above guidelines. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

The purpose of this study was to evaluate any effects of bicyclopyrone (NOA449280, purity 94.5%) on the pregnant female rat and development of the embryo and foetus consequent to exposure to bicyclopyrone from day 5 (implantation) to day 20 post coitum (the day prior to Caesarean section). The results of this study were used to establish suitable dose levels for a subsequent prenatal developmental toxicity study in the rat.

Bicyclopyrone was administered by oral gavage to 10 mated female RccHan: WIST(SPF) in 0.5% carboxymethyl cellulose (CMC) in purified water at dose levels of 0, 100, 500 and 1000 mg/kg bw/day on days 5 to 20 of gestation.

All females were sacrificed on day 21 post coitum and the foetuses were removed by Caesarean section. Examination of dams and foetuses was performed in accordance with international recommendations.

All female animals survived until scheduled necropsy. At 500 and 1000 mg/kg/day, individual dams were noted moving their heads through the bedding material. This was considered to be a non-specific sign of discomfort.

At 500 and 1000 mg/kg/day, mean food consumption was statistically significantly reduced between days 5 to 17 and days 5 to 14, respectively. Mean food consumption at 100 mg/kg/day was slightly, but statistically significantly, lower than control on gestation days 5-8.

Statistically significantly lower maternal body weights and/or maternal body weight gains were observed in all dose groups when compared to the controls, which paralleled the lower

food consumption in these groups. At 500 and 1000 mg/kg/day, maternal body weights were beginning to diverge from control values on days 9 and 7, respectively. Mean weight gain was affected as early as gestation day 6 in all dose groups. However, these effects were transient and by gestation day 21, both body weight and weight gain were comparable across all dose groups.

There was no effect on pre-implantation loss, implantation rate, post-implantation loss or the number of live foetuses. There were no treatment-related effects at ophthalmological examination or scheduled necropsy.

Based upon the effects in this study, the maternal NOAEL is 1000 mg/kg/day. The maternal LOAEL was not observed.

There were no effects on foetal sex ratios. At 1000 mg/kg/day, the mean body weight of foetuses was statistically significantly reduced. At scheduled Caesarean section, no treatment-related macroscopic findings were noted in the pups. Numerous visceral variations were observed scattered across all dose groups, including controls. Though the overall incidence of visceral variations appeared to be higher in the 500 and 1000 mg/kg/day dose groups compared to controls, the vast majority of these were common variations in the rat and were within the historical control ranges.

There were no skeletal abnormalities observed in any dose group.

Statistically significant increases in the incidence of pelvic girdle displaced (caudal), also known as 27 pre-sacral vertebrae, were observed in all dose groups when compared to controls. In addition, increases in the incidence of costal cartilages asymmetrically aligned at the sternum were observed at 500 and 1000 mg/kg/day. All other skeletal variations were considered to reflect the normal variability in this species.

Increases in the litter incidences of non-ossified cervical vertebrae and the presence of supernumerary and/or rudimentary ribs were observed in all dose groups when compared to controls. The litter incidence of long costal cartilage 11 was increased in all dose groups compared to the concurrent controls. In addition, the litter incidence of supernumerary costal cartilage was increased at 1000 mg/kg/day. All other cartilage variations were considered to reflect the normal variability in this species.

Based upon the effects in this study, the developmental LOAEL is 100 mg/kg/day based upon skeletal and cartilage effects. The developmental NOAEL was not observed.

This study is classified totally reliable **(acceptable/non-guideline)** and satisfies the registrants' need (OPPTS 870.3700a; OECD 414) for a range-finding study developmental toxicity study in rats. APVMA as a matter of policy does not set LOAELs for developmental range-finding studies. Furthermore, APVMA's view is that the variations described (caudal displacement of the pelvic girdle, supernumerary ribs, ossification delays and cartilage changes) do not represent a structural defect (i.e. are not teratogenic effects), and have no functional deficit, noting that the two-generation reproduction study did not find reproductive toxicity potential associated with the test material. On this basis, while noting that the skeletal findings are treatment-related, an increase in these variations alone is not of sufficient severity that the test material should be classified as a developmental toxicant. APVMA and

PMRA further believe that the treatment related effect in foetuses may in part be due to the small number of foetuses examined.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar J. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/17/2015
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Prenatal Developmental Study (rabbit). OECD 414 (2001): OPPTS 870.3700 (1998): EC 2004/73 B.31 (2004): JMAFF 12 NohSan No. 8147 (2000)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Coder P, 2012. NOA449280: A prenatal developmental toxicity study in New Zealand White rabbits. WIL Research Laboratories, LLC, 1407 George Road, Ashland, OH 44805-8946 USA. Laboratory Report No. WIL-639040, 10 September 2012. Unpublished. Syngenta File No. NOA449280/11297. MRID 47841996

SPONSOR: Syngenta Crop Protection, LLC, 410 Swing Road, Post Office Box 18300, Greensboro, NC 27409-8300 USA

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a prenatal developmental toxicity study in rabbits (MRID #47841996), bicyclopyrone (NOA449280, 94.5%), in the vehicle, 0.5% (w/v) aqueous high viscosity carboxymethylcellulose (CMC), was administered orally by gavage to 3 groups of 25 time-mated young adult, female New Zealand White [Hra:(NZW)SPF] rabbits once daily from gestation days 7 through 28. Dose levels were 10, 50, and 200 mg/kg/day administered at a dose volume of 10 mL/kg. A concurrent control group composed of 25 time-mated females received the vehicle (0.5% high viscosity CMC) on a comparable regimen. The females were approximately 5.5 months of age at the initiation of dose administration. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights, and food consumption were recorded at appropriate intervals. Blood samples for plasma analysis of parent test substance and tyrosine concentrations were collected from all surviving females at approximately 6 hours following dose administration on gestation day 28. A laparohysterectomy was performed on each surviving female on gestation day 29. The uteri, placentae, and ovaries were examined, and the numbers of foetuses, early and late resorptions,

total implantations, and *corpora lutea* were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The foetuses were weighed, sexed, and examined for external, visceral, and skeletal malformations and developmental variations.

Maternal effects are as follows:

There were no test substance-related effects on survival or clinical or macroscopic findings for maternal animals at 10 and 50 mg/kg/day. Mean maternal body weight and food consumption parameters were unaffected at 10 and 50 mg/kg/day.

A 200 mg/kg/day bicyclopyrone, significant maternal toxicity was manifested with 7 females in this group found dead (including 1 female that was found dead following an abortion) or killed *in extremis* from gestation day 22 to 28 following food consumption of ≤10 g/day for at least 4 days prior to death or euthanasia, and body weight losses (as much as 639 g from the gestation day 7 body weight). An additional 2 females in the 200 mg/kg/day group delivered on gestation day 29 following slight reductions in food consumption or body weight loss. There were not effects on absolute maternal body weights. Mean maternal body weight gains for this group were lower (although not statistically significantly) than the control group during gestation days 13-21 and a mean body weight loss was noted during gestation days 21-29. Although mean food consumption values and mean body weights were not remarkably different from control group values, the effects on body weight gains often occurred in conjunction with low food consumption and decreased defecation when evaluated on an individual animal basis. Mean gravid uterine weight, net body weight, and net body weight gain for surviving females were unaffected at this dose level. There were no test substance-related macroscopic findings observed in the 200 mg/kg/day group.

Based on mortality/moribundity in conjunction with minimal food consumption and effects on body weight changes, the maternal LOAEL is 200 mg/kg/day group. The maternal NOAEL is 50 mg/kg/day.

Fetal effects are as follows:

Test substance-related, statistically significant increases in a number of skeletal variations were observed in all test substance-treated groups compared to the control group. These included increased incidences of 13th full rib(s), 27 presacral vertebrae, and an extra site of ossification ventral to cervical centrum no. 2.

A low incidence of skeletal costal cartilage anomalies (fused, bifurcated, and/or malpositioned costal cartilage) was observed in the 200 mg/kg/day group (4 foetuses from 3 litters in the 200 mg/kg/day group compared to 0 foetuses in the control group). In addition, 2 foetuses from 2 litters at 50 mg/kg/day also exhibited costal cartilage anomalies. Although these values were at the upper level of the historical control incidence, an association with the test substance cannot be excluded.

Based on the effects in this study, the developmental LOAEL is 10 mg/kg/day based upon skeletal variations (the appearance of 13th full rib, and the 27th presacral vertebrae). The developmental NOAEL was not observed.

This study is classified totally reliable (**acceptable/guideline**) and satisfies the intent of the guideline requirement (OPPTS 870.3700b; OECD 414) for a developmental toxicity study in rabbits.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Technical, light brown powder
Lot/Batch number:	SEZ3AP006/MILLED
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Stability confirmed (stored at a temperature < 30°C). Reanalysis date March 2011

Structures:

Vehicle and/or positive control: The test substance was administered in 0.5% w/v aqueous carboxymethylcellulose (CMC), high viscosity.

Test Animals:

Species	Rabbit
Strain	Hra:(NZW)SPF
Age/weight at dosing	Approximately 5 ½ months/2900-4500 g
Source	Covance Research Products, Inc., Kalamazoo, MI, USA
Housing	Individually in clean, stainless steel suspended cages
Acclimatisation period	The time-mated rabbits were received on gestation day 1, 2 or 3
Diet	Certified Rabbit LabDiet® 5322 (PMI Nutrition International, LLC). The basal diet was offered in 25 g increments 3 times per day on the day of arrival and in increased amounts over the next few days, until the animals gradually achieved ad libitum status prior to the dose administration period; basal diet was offered ad libitum thereafter.
Water	Reverse osmosis-purified (on-site) drinking water ad libitum
Environmental conditions	Temperature: 18.3-19.3°C Humidity: 48.6-61.6% Air changes: Minimum of 10 changes/hour Photoperiod: 12 hours light/12 hours dark

Study Design and Methods:

In-life dates: Start: 16 December 2008 (first day of dosing) End: 09 January 2009

Mating procedure: The time-mated rabbits were received on gestation day 1, 2 or 3; a breeding record was provided by the supplier.

Animal assignment: Animals were randomly assigned to test groups by a computerised randomisation procedure as shown in the following table.

Table 1: Animal numbers and treatment groups

Group	Test substance	Dose level (mg/kg/day)	Test substance concentration (mg/mL)	Dose volume (mL/kg)	Number of females
1	Vehicle (CMC)	0	0	10	25
2	NOA4499280	10	1	10	25
3	NOA4499280	50	5	10	25
4	NOA4499280	200	20	10	25

Table was taken from page 16 of the study report

Dose selection rationale: Dose levels were determined from results of previous studies. In an initial dose range-finding study (***Sawhney Coder; 2012***, WIL-639027), dose levels of 250 and 400 mg/kg/day resulted in excessive maternal toxicity leading to early group termination (See Appendix 1). In a follow-up range-finding study (***Sawhney Coder; 2012***, WIL 639034), maternal effects on body weight and reduced foetal weight were noted at 200 mg/kg/day (See Appendix 2). Based on these results, dose levels of 10, 50 and 200 mg/kg/day were selected for evaluation in the current study.

Dosage preparation and analysis: The test substance suspensions were weight/volume (test substance/vehicle) mixtures. The test substance suspensions were prepared every 4 days as single preparations for each dose level, divided into aliquots for daily dispensation, and stored refrigerated (approximately 4ºC). The test substance suspensions were stirred continuously throughout the preparation, sampling, and dose administration procedures. The pH measurements of the first preparations were 7.66, 5.36, 4.15, and 4.11 for the 0, 1, 5, and 20 mg/mL suspensions, respectively.

The first dosing suspensions were visually inspected and were found to be visibly homogeneous and acceptable for administration.

Concentration analysis results: Achieved concentrations were within 90%-110% of nominal.
Homogeneity results: The RSD for the mean concentration was 5% or less at a concentration within the acceptable limits (90% to 110% of target).
Stability: The stability (following 5 days of refrigerated storage) of bicyclopyrone (NOA449280) in 0.5% CMC was confirmed.

The analytical data indicate that the test substance was homogeneous and stable in the suspensions and that the variation between the nominal and actual dosages to the animals was acceptable.

Dosage administration: The vehicle and test substance suspensions were administered orally, by gavage, once daily during gestation days 7-28. The dose volume for all groups was 10 mL/kg. Individual doses were based on the most recently recorded body weights to provide the correct mg/kg/day dose.

Observations:

Maternal observations: All rabbits were observed twice daily, once in the morning and once in the afternoon, for moribundity and mortality. Individual detailed clinical observations were recorded from the day of receipt through gestation day 29 (prior to dose administration during the treatment period). Animals were also observed for signs of toxicity approximately 4 hours following dose administration.

Individual maternal body weights were recorded on gestation days 0 (by the supplier), 4, and 7-29 (daily). Group mean body weights were calculated for each of these days. Mean body weight changes were calculated by cumulatively normalising daily body weight change from the first day of dose administration (gestation day 7), and for gestation days 0-4, 0-7, 7-10, 10-13, 13-21, and 21-29.

Gravid uterine weight was collected and net body weight (the gestation day 29 body weight exclusive of the weight of the uterus and contents) and net body weight change (the gestation day 0-29 body weight change exclusive of the weight of the uterus and contents) were calculated and presented for each gravid female at the scheduled laparohysterectomy.

Individual food consumption was recorded on gestation days 4-29 (daily). Food intake was reported as g/animal/day for the corresponding body weight change intervals.

Blood samples (approximately 3 mL each) for plasma analysis of parent test substance and L-tyrosine concentrations were collected from all surviving females on gestation day 28 at approximately 6 hours following dose administration. Blood was collected via a marginal ear vein into chilled tubes containing lithium heparin as the anticoagulant. Samples were maintained on wet ice and protected from light until processed in a refrigerated centrifuge (at approximately 4ºC). Plasma was transferred to new, uniquely labelled polypropylene tubes, flash frozen in liquid nitrogen, and stored frozen (approximately -70ºC) until analysis by WIL Bioanalytical Chemistry Department.

A gross necropsy was performed on females that died, were killed *in extremis* or aborted during the course of the study. Maternal tissues were retained in 10% neutral-buffered formalin for possible future histopathological examination only as indicated by the gross findings. The number and location of implantation sites, *corpora lutea*, and viable foetuses were recorded. Recognisable foetuses were examined externally and retained in 10% neutral-buffered formalin. The females and all other products of conception were then discarded.

The scheduled laparohysterectomies and macroscopic examinations were performed blind to treatment group. All rabbits were killed on gestation day 29 by an intravenous injection of sodium pentobarbital via the marginal ear vein. The thoracic, abdominal and pelvic cavities were opened by a ventral mid line incision, and the contents were examined. In all instances, the post mortem findings were correlated with the ante mortem comments, and any abnormalities were recorded. The uterus and ovaries were then exposed and excised. The number of *corpora lutea* on each ovary was recorded. The trimmed uterus was weighed (with the exception of the females that delivered) and opened, and the number and location of all foetuses, early and late resorptions and the total number of implantation sites were recorded. The placentae were also examined. The individual uterine distribution of implantation sites was documented using the following procedure. All implantation sites, including resorptions, were numbered in consecutive order beginning with the left distal to the left proximal uterine

horn, noting the position of the cervix, and continuing from the right proximal to the right distal uterine horn.

Maternal tissues were preserved for possible future histopathological examination only as indicated by the gross findings. Representative sections of corresponding organs from a sufficient number of control animals were retained for comparison. The carcass of each female was then discarded.

Uteri with no macroscopic evidence of implantation were opened and subsequently placed in 10% ammonium sulfide solution for detection of early implantation loss (***Salewski, 1964***).

Foetal observations: Each foetus and delivered kit was examined externally, individually weighed, killed by hypothermia followed by an intrathoracic injection of sodium pentobarbital (if necessary) and tagged for identification. The detailed external examination of each foetus included, but was not limited to, an examination of the eyes, palate and external orifices, and each finding was recorded. Crown rump measurements, degrees of autolysis and gross examinations, if possible, were recorded for late resorptions, and the tissues were discarded.

Each foetus was subjected to a visceral examination using a modification of the Stuckhardt and Poppe fresh dissection technique to include the heart and major blood vessels (***Stuckhardt and Poppe, 1984***). The sex of each foetus was determined by internal examination. Foetal kidneys were examined and graded for renal papillae development (***Woo and Hoar, 1972***). Heads from all foetuses were examined by a mid-coronal slice. All carcasses were eviscerated and fixed in 100% ethyl alcohol.

Following fixation in alcohol, each eviscerated foetus was macerated in potassium hydroxide and stained with Alizarin Red S and Alcian Blue by a method similar to that described by ***Dawson (1926)*** and ***Inouye (1976)***. The exact procedure was based on an evaluation study conducted by WIL Research Laboratories (***Sawhney Coder, 2008; WIL-99458***). External, visceral and skeletal findings were recorded as developmental variations (alterations in anatomic structure that are considered to have no significant biological effect on animal health or body conformity and/or occur at high incidence, representing slight deviations from normal) or malformations (those structural anomalies that alter general body conformity, disrupt or interfere with normal body function, or may be incompatible with life).

Statistical analyses: Analyses were conducted using two-tailed tests, comparing each test substance-treated group to the control group. Where applicable, the litter was used as the experimental unit. Mean maternal body weights, body weight changes, and food consumption, gravid uterine weights, numbers of *corpora lutea*, implantation sites, viable foetuses, and mean foetal body weights (separately by sex and combined) were subjected to a parametric one-way analysis of variance (ANOVA) followed by Dunnett's test.

Maternal performance data and macroscopic findings were analysed by Fisher's Exact Test, comparing each test substance-treated group to the control group. The incidence of foetal malformations and developmental variations were summarised as the proportion of foetuses affected and the proportion of litters affected. The proportions were analysed by a two-tailed Fisher's Exact Test, comparing each test substance-treated group to the control group.

Mean litter proportions (percent per litter) of prenatal data (viable and nonviable foetuses, early and late resorptions, total resorptions, pre- and postimplantation loss and foetal sex distribution), were summarised as the group proportion affected. The litter based mean percentage was summarised and subjected to a double arcsine transformation (***Freeman and Tukey, 1950***) followed by ANOVA and Dunnetts test.

WIL Research Laboratories, LLC has historical control data on the background incidence of foetal malformations and developmental variations in this species from the same strain and source.

RESULTS

Maternal toxicity:

Mortality and clinical signs: A dose of 200 mg/kg/day exceeded the maximum tolerated dose (MTD) in the New Zealand White rabbit. Significant maternal toxicity was manifested at a dose level of 200 mg/kg/day, with 7 females in this group found dead (including 1 female that was found dead following an abortion) or killed *in extremis* from gestation day 22 to 28 following food consumption of ≤10 g/day for at least 4 days prior to death or euthanasia and body weight losses. An additional 2 females in the 200 mg/kg/day group delivered on gestation day 29 following slight reductions in food consumption or body weight loss.

There were no test substance-related effects on survival or clinical findings for maternal animals at 10 and 50 mg/kg/day. The increased incidences of death and sacrifices in extremis of rabbits in the control, 10, and 50 mg/kg/day groups were not test substance related.

Body weight: There were no treatment related effects on absolute maternal body weights. Mean maternal body weight gains for the 200 mg/kg/day group were lower (although not statistically significantly) than the control group during gestation days 13-21 and a mean body weight loss was noted during gestation days 21-29. Although mean body weights were not remarkably different from control group values, the effects on body weight gains often occurred in conjunction with low food consumption and decreased defecation when evaluated on an individual animal basis.

Mean maternal body weight parameters were unaffected at 10 and 50 mg/kg/day. See tables 2 and 3.

Table 2: Summary of absolute body weights during gestation (g) (selected time points)

	Dose level of bicyclopyrone (mg/kg/day)			
days	**0 (control)**	**10**	**50**	**200**
0	3766 ± 265.2	3735 ± 286.5	3685 ± 320.0	3695 ± 331.3
7	3820 ± 270.0	3803 ± 296.9	3770 ± 306.8	3788 ± 291.0
12	3843 ± 282.6	3813 ± 298.4	3792 ± 323.6	3831 ± 323.7
17	3910 ± 309.8	3900 ± 337.9	3890 ± 360.4	3837 ± 379.8
22	3961 ± 336.6	3898 ± 314.9	3933 ± 381.1	3870 ± 397.3
29	3925 ± 329.1	3910 ± 296.4	3925 ± 295.5	3767 ± 331.9

Data were taken from pages 40-44 of the study report

Table 3: Summary of body weight change during gestation (g) (selected time points)

	Dose level of bicyclopyrone (mg/kg/day)			
days	**0 (control)**	**10**	**50**	**200**
0-7 (pre-treatment)	53 ± 87.6	68 ± 84.1	86 ± 107.5	93 ± 97.7
7-12	24 ± 69.1	9 ± 58.9	22 ± 61.3	42 ± 70.7
7-17	90 ± 143.6	96 ± 110.1	111 ± 118.1	49 ± 197.7
7-22	141 ± 198	120 ± 193.7	154± 158.6	94 ± 299.5
7-29	105 ± 216.2	139 ± 243.8	129± 190.1	87 ± 209.9

Data were taken from pages 45-50 of the study report

Table 4: Females Demonstrating Minimum Food Consumption

	Dose level of bicyclopyrone (mg/kg/day)			
			0	**200**
No. of females with minimum food consumption (gestation days 21-29) 4 days of longer in duration			6	13
No. of females with minimal food consumption found dead, euthanized in extremis, delivered, or aborted			1	8

Data were taken from page 27 of the study report

Food consumption: Mean maternal food consumption, evaluated as g/animal/day, in the 10, 50, and 200 mg/kg/day groups was similar to the control group throughout the treatment period (gestation days 7-10, 10-13, 13-21, 21-29, and 7-29). Food consumption was periodically reduced in individual animals throughout the study; these reductions were most evident during the latter part of gestation (gestation days 21-29), the period in which test substance-related mortality was noted in the 200 mg/kg/day group. Although the reductions for the individual 200 mg/kg/day group females were not sufficient to affect the overall group means, the reduced food consumption was considered test substance related due to the number of females affected and the resultant mortality/moribundity in this group. See table 5.

Table 5: Summary of food consumption (g/animal/day) (selected time points)

	Dose level of bicyclopyrone (mg/kg/day)			
day	**0 (control)**	**10**	**50**	**200**
4-7	153 ± 25.4	158 ± 30.9	162 ± 30.8	163 ± 33.2
7-10	148 ± 25.3	148 ± 24.4	149 ± 33.7	157 ± 27.8
7-29	112 ± 37.2	115± 31.9	112 ± 26.0	118 ± 28.3

Data were taken from pages 53-58 of the study report

Plasma analyses: The measured bicyclopyrone concentrations were below limit of quantification, 116-31000, 1090-112000 and 9770-325000 ng/mL at dose levels of 0, 10, 50 and 200 mg/kg/day respectively. The measured L-tyrosine concentrations were below limit of quantification, 29.7-150, 40.6-92.0 and 51.1-157 µg/mL at dose levels of 0, 10, 50 and 200 mg/kg/day respectively.

Sacrifice and pathology:
Maternal gross pathology: There were no test substance-related macroscopic findings at any dose level.

Caesarean section data: Data are summarised in the table below in table 6. In surviving females, live litter size, mean fetal body weights, fetal sex ratios, mean number of corpora lutea and implantation sites, and the mean litter proportions of pre- and post-implantation loss were similar across all groups. Differences from the control group were slight and not statistically significant.

Table 6: Summary of Caesarean section data

Observation	Dose level of bicyclopyrone (mg/kg bw/day)			
	0 (control)	10	50	200
Number of dams (gravid)	25 (23)	25 (23)	25 (23)	25 (18)
Females that aborted or delivered	0	1	0	0
Females that died	1	1	1	5
Pregnant females killed *in extremis*	0	0	1	2
Corpora lutea: total number (mean per dam)	241 (10.5)	222 (9.7)	241 (10.5)	180 (10.6)
Resorptions (total) - early	6	7	7	2
Resorptions (% per litter) - early	2.6	4.0	3.5	1.1
Resorptions (total)- late	7	5	9	7
Resorptions (% per litter) - late	2.9	2.4	4.0	4.2
Total resorptions (% of litter)	5.5	6.3	7.5	5.4
Pre-implantation loss: total number (mean per dam)	16 (0.7)	27 (1.2)	14 (0.6)	17.0 (1.0)
Pre-implantation loss: (% of litter)	6.6	12.1	5.6	9.8
Post-implantation loss: total number (mean per dam)	13 (0.6)	12 (0.5)	16 (0.7)	16 (0.9)
Post-implantation loss (% of litter)	5.5	6.3	7.5	8.9
Implantation sites: total	225	195	227	172
Live foetuses (total)	212	183	211	156
Live foetuses (% of litter)	94.5	93.7	92.5	91.1
Dead foetuses: total number (% of litter)	0 (0)	0 (0)	0 (0)	7 (3.5)
Number of male foetuses	91	87	108	75
(% of foetuses)	42.5	49.0	51.5	48.5
Number of female foetuses	121	96	103	81
Mean wt (g) of live foetuses (litter basis) (total)	36.4 ± 6.05	39.4 ± 7.71	38.1 ± 5.48	35.2 ± 6.81
Mean wt (g) of live foetuses (litter basis) (male)	36.6 ± 6.67	39.7 ± 7.82	38.4 ± 5.61	36.0 ± 7.11
Mean wt (g) of live foetuses (litter basis) (female)	35.9± 6.12	39.3 ± 8.07	37.7± 5.63	34.4 ± 7.92

Data were taken from pages 63-65

Developmental toxicity: Intrauterine foetal growth and survival were unaffected by test substance administration at dose levels of 10, 50, and 200 mg/kg/day for females that survived to gestation day 29. See table 7.

The numbers of foetuses (litters) available for morphological evaluation were 212(23), 183(23), 211(23), and 156(18) in the control, 10, 50, and 200 mg/kg/day groups, respectively. Malformations were observed in 2(2), 1(1), 6(5), and 5(4) foetuses (litters) in these same respective dose groups.

External examinations: External malformations were noted in 2 foetuses from a single litter in the 200 mg/kg/day group and 1 foetus in the 50 mg/kg/day group. Two foetuses from the same litter in the 200 mg/kg/day group were noted with bilateral microphthalmia with no changes in the size of the ocular orbital. This finding was considered not to be test substance-related because it occurred in a single litter and the mean proportion of foetuses affected was not significantly different from the control group. One foetus in the 50 mg/kg/day group was noted with meningoencephalocele, where a portion of the meninges and brain protruded through an opening in the anterior cranium. Due to the lack of a dose response (finding not observed at 200 mg/kg/day), this finding was considered not to be test substance-related. There were no external malformations for foetuses in the control or 10 mg/kg/day groups.

The only external developmental variation was 1 occurrence of twinning in the 200 mg/kg/day group.

Visceral examinations: Soft tissue malformations were noted for 1(1), 1(1), 1(1) and 2(2) foetuses (litters) in the control, 10, 50, and 200 mg/kg/day groups, respectively. No statistically significant differences from the control group were noted.

A non-dose-responsive higher incidence of absent or small gallbladder was noted in the test substance-treated groups when compared to the control group. Although the higher incidences at 10 and 200 mg/kg/day were statistically significantly different from the control group, this finding is relatively common in laboratory rabbits and was observed similarly in the WIL historical control data. Decreases in the numbers of foetuses with an extra papillary muscle and major blood vessel variation were noted in the test substance-treated groups; the decreased incidences in the 200 mg/kg/day group were statistically significant. Decreases in extra papillary muscle and major blood vessel variation are not of toxicological concern. All other visceral variations noted in the test substance-treated groups were observed in single animals, in a pattern similar to the control group, and/or in a manner that was not dose responsive.

An accessory lobule of the liver was observed in 2 foetuses in the control and 10 mg/kg/day groups, respectively). In addition, renal papillae not fully developed was noted in 2 foetuses in the 10 and 200 mg/kg/day groups), and a depressed area of the posterior cranium was observed for one foetus in the 200 mg/kg/day group. These findings were not classified as either malformations or developmental variations but were considered a reflection of normal variability, and were not included in any tabulation.

Skeletal examinations: A low incidence of skeletal costal cartilage anomalies (fused, bifurcated, and/or malpositioned costal cartilage) was observed in the 200 mg/kg/day group (3 foetuses from 2 litters in the 200 mg/kg/day group compared to 0 foetuses in the control group). In addition, 2 foetuses from 2 litters at 50 mg/kg/day also exhibited costal cartilage anomalies. Although these values were at the upper level of the historical control incidence (1.1%), an association with the test substance cannot be excluded.

Test substance-related, statistically significant increases in a number of skeletal variations were observed in all test substance-treated groups compared to the control group. These included increased incidences of 13th full rib(s), 27 presacral vertebrae, and an extra site of ossification ventral to cervical centrum no. 2. The study authors considered these skeletal variations to be non-adverse.

Other skeletal developmental variations observed in the test substance-treated groups occurred similarly in the control group, in single foetuses, and/or in a manner that was not dose-related.

Table 7: Intergroup comparison of treatment-related findings in foetuses (litters)

	Dose level of Bicyclopyrone (mg/kg/day)			
	0	**10**	**50**	**200**
Number of foetuses (litters) examined	212 (23)	183 (23)	211 (23)	156 (18)
Skeletal anomalies				
Costal cartilage anomaly	0 (0)	0 (0)	2 (2)	3 (2)
Skeletal variations				
13th full rib	51 (17)	112** (21)	149** (23)*	147** (18)*
27 presacral vertebrae	10 (7)	53** (15)*	92** (21)**	110** (18)**
Extra site of ossification ventral to cervical centrum no. 2	0 (0)	3 (3)	4 (1)	4* (1)
* sig difference from control at 0.05 (Fisher's Exact Test) ** sig difference from control at 0.01 (Fisher's Exact Test)				

Data were taken from pages 71-78

INVESTIGATOR'S CONCLUSIONS:

Based on mortality/moribundity in conjunction with minimal food consumption and effects on body weight changes observed in the 200 mg/kg/day group, a dose level of 50 mg/kg/day was considered to be the no-observed-adverse-effect level (NOAEL) for maternal toxicity.

Based on the lack of evidence for any significant developmental toxicity at 10 mg/kg/day, the NOAEL for prenatal developmental toxicity for bicyclopyrone when administered orally by gavage to pregnant New Zealand White rabbits was 10 mg/kg/day.

REVIEWER'S COMMENTS:

Based on mortality/moribundity in conjunction with minimal food consumption and effects on body weight changes, the maternal LOAEL is 200 mg/kg/day group. The maternal NOAEL is 50 mg/kg/day.

Based on the effects in this study, the developmental LOAEL is 10 mg/kg/day based upon skeletal variations (the appearance of 13th full rib, and the 27th presacral vertebrae). The developmental NOAEL was not observed.

This study is classified totally reliable (**acceptable/guideline**) and satisfies the guideline requirement (OPPTS 870.3700b; OECD 414) for a developmental toxicity study in rabbits. EPA, PMRA (Canada), APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

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(Coder P, 2012)

Appendix 1

STUDY TYPE: Dose Range-finding Prenatal Developmental Study (rabbit). OPPTS 870.3700 (1998)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Moxon M, 2007. NOA449280: A dose range finding study in the pregnant rabbit. Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK. Laboratory Report No. RB1092-REG, 17 July 2007. Unpublished. Syngenta File No. NOA449280/0049.MRID 47841994

SPONSOR: Syngenta Limited, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

Groups of 10 time-mated, female New Zealand White rabbits were dosed orally, by gavage, with 0 (control), 100, 200 or 500 mg bicyclopyrone/kg/day (purity 94.5%) (using 0.5% w/v aqueous carboxymethylcellulose as a vehicle) on days 5-29 (inclusive) of gestation. The day of mating was designated day 1 of gestation. The rabbits were terminated on day 30 of gestation with the exception of those given 500 mg/kg/day which were terminated prematurely due to signs of overt toxicity.

The following observations and measurements were made: Dams - Clinical observations, body weights, food consumption, plasma concentrations of bicyclopyrone, number of *corpora lutea*, gravid uterus weight. Foetuses/litters - Number and position of implantations, number of live foetuses, number of intra-uterine deaths (early/late), foetal weight, foetal sex, external and visceral observations, skeletal (bone and cartilage) observations.

Two rabbits given 500 mg/kg/day died or were killed due to poor clinical condition on day 8. In addition, 3 of the remaining rabbits given 500 mg/kg/day had clinical signs including pinched in sides, reluctance to move, irregular breathing and subdued behaviour. As it was clear that the dose level of 500 mg/kg/day could not be tolerated for the duration of the study, all surviving rabbits given this dose level were terminated on day 7 or 8 of gestation. Macroscopic changes were observed in the stomachs of the rabbits and included ulceration, red spot/s and area/s and, sloughing on the mucous layer.

The dose levels of 100 and 200 mg/kg/day were associated with lower maternal body weights but there was no dose-response and no effect on maternal food consumption or clinical condition.

Based upon the effects of this study, the maternal LOAEL is 500 mg/kg/day based upon clinical signs. The maternal NOAEL is 200 mg/kg/day.

Systemic exposure of the rabbits to bicyclopyrone was confirmed from plasma analysis. Average concentrations of bicyclopyrone in plasma increased in proportion with dose and were similar on days 17 and 29 of gestation.

There was no effect of 100 or 200 mg/kg/day on the number, growth or survival of the foetuses *in utero* and no evidence for any effect on the type or incidence of foetal abnormality. There was however, a treatment-related increase in the incidence of 3 specific skeletal variations: a lengthened costal cartilage on rib 10, rib 13 (extra rib) of long length attached to the vertebral column and 27 pre-pelvic vertebrae.

Based upon the effects of this study, the developmental LOAEL is 100 mg/kg/day based upon an increased incidence of skeletal variations. The developmental NOAEL is not established.

This study is classified totally reliable **(acceptable/non-guideline)** and satisfies the intent of the guideline requirement (OPPTS 870.3700b; OECD 414) for a range-finding developmental toxicity study in rabbits.

Appendix 2

STUDY TYPE: Dose Range-finding Prenatal Developmental Study (rabbit). Adapted from OECD 414 (2001): OPPTS 870.3700 (1998): EC 2004/73 B.31 (2004): JMAFF 12 NohSan No. 8147 (2000)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Coder P, 2012. NOA449280: A dose range-finding prenatal developmental toxicity study in New Zealand White rabbits. WIL Research Laboratories, LLC, 1407 George Road, Ashland, OH 44805-8946 USA. Laboratory Report No. WIL-639027, 23 August 2012. Unpublished. Syngenta File No. NOA449280/50287. MRID 47841995

SPONSOR: Syngenta Crop Protection, LLC, 410 Swing Road, Post Office Box 18300, Greensboro, NC 27409-8300 USA

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There are no regulatory test guidelines for developmental toxicity range-finding studies. The study procedures were adapted from the current guidelines for prenatal developmental studies. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

The test substance, Bicyclopyrone (NOA449280, purity 94.5%), in the vehicle, 0.5% (w/v) aqueous high viscosity carboxymethylcellulose (CMC), was administered orally by gavage to 4 groups of 8 time-mated female New Zealand White [Hra:(NZW)SPF] rabbits once daily from gestation days 7 through 28. Dose levels were 10, 50, 250, and 400 mg/kg/day administered at a dose volume of 10 mL/kg. A concurrent control group composed of 8 time-mated females received the vehicle (0.5% high viscosity CMC) on a comparable regimen.

All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights, and food consumption were recorded at appropriate intervals. Blood samples for plasma analysis of bicyclopyrone, NOA454598 (desmethyl metabolite), and L-tyrosine were collected from all females on gestation day 7 and from the surviving females on gestation days 17 and 28 at approximately 2 hours following dose administration. On gestation day 29, a laparohysterectomy was performed on each surviving female. The uteri, placentae and ovaries were examined, and the numbers of foetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The foetuses were weighed, sexed, and examined for external, visceral, and skeletal malformations and developmental variations.

Maternal toxicity was observed in the 250 and 400 mg/kg/day groups as evidenced by mortality, moribundity, abortion, and early group termination. Mean body weight losses

and/or lower mean body weight gains with corresponding reduced food consumption (differences from the control group not statistically significant) were noted in these same groups. Early group termination in the 250 and 400 mg/kg/day groups precluded evaluation of intrauterine parameters and foetal morphology. There were no test substance-related changes noted macroscopically for females found dead, killed *in extremis* or that aborted in the 250 and 400 mg/kg/day groups or for females in the 10 and 50 mg/kg/day groups that survived to the scheduled necropsy.

Analysis of plasma samples collected during gestation revealed consistent dose-proportional increases in the plasma levels of parent bicyclopyrone throughout gestation, with plasma levels of the NOA454598 metabolite generally present at approximately 5% of parent levels.

Plasma tyrosine levels measured on gestation day 7 were elevated at all dose levels, reaching a plateau even at the low dose of 10 mg/kg/day. Subsequent analyses revealed plasma tyrosine levels continued to increase at 250 mg/kg/day (the high dose having been terminated prior to further collection).

Based on mortality/moribundity in conjunction with minimal food consumption and effects on body weight changes, the maternal LOAEL is 250 mg/kg/day group. The maternal NOAEL is 50 mg/kg/day.

Intrauterine growth and survival in the 10 and 50 mg/kg/day groups were similar to the control group. High mean litter proportions of 13th full rib (s) and 27 presacral vertebrae were noted in the 50 mg/kg/day group compared to the control group. Foetal morphology in the 10 mg/kg/day group was similar to the control group.

Based upon the effects in this study, the developmental NOAEL is 10 mg/kg/day. The developmental LOAEL is 50 mg/kg/day based upon higher mean litter proportions of 13th full rib (s) and 27 presacral vertebrae were noted in the 50 mg/kg/day group compared to the control group.
This study is classified totally reliable (**acceptable/non-guideline)** and satisfies the intent of the guideline requirement (OPPTS 870.3700b; OECD 414) for a range-finding developmental toxicity study in rabbits.

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar J. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/19/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/19/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Prenatal Developmental Study (rabbit). OECD 414 (2001): OPPTS 870.3700 (1998): JMAFF 12NohSan No. 8147 (2000)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Whitlow S, 2012. NOA449280: Prenatal developmental toxicity study in the Himalayan rabbit. Harlan Laboratories Ltd., Wölferstrasse 4, 4414 Füllinsdorf, Switzerland. Laboratory Report No. C41898, 19 September 2012. Unpublished. Syngenta File No. NOA449280/11299.MRID 47841998

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a developmental toxicity study in Himalayan rabbits (MRID #47841998), bicyclopyrone was administered to pregnant Himalayan rabbits. The effects on development of the embryo and foetus consequent to exposure of the female to the test item from day 6 *post coitum* (implantation) to day 27 *post coitum* (the day prior to Caesarean section) were determined.

Each group consisted of 22 mated young adult female Himalayan rabbits. Bicylopyrone was administered at dose levels of: 0 (vehicle control), 10, 50 or 250 mg/kg/day. A standard dose volume of 4 mL/kg body weight with a daily adjustment to the actual body weight was used. Control animals were dosed with the vehicle alone (0.5% Carboxymethyl cellulose high viscosity). At scheduled sacrifice on day 28 *post coitum*, the females were killed and the foetuses were removed by Caesarean section. Examination of dams and foetuses was performed in accordance with international recommendations.

The maternal effects are as follows:

At 10 mg/kg/day, there were no treatment-related maternal effects. At 50 mg/kg/day, a single dam aborted her litter. There were no mortalities, obvious signs of maternal toxicity noted in any dose group. There were no significant effects on body weight gains or food consumption, and no mortality was observed. Measurement of haematology and clinical chemistry parameters did not indicate any toxicologically significant changes.

At 250 mg/kg/day, necropsy examination of maternal animals revealed a low number of animals with macroscopic findings of the stomach wall suggesting irritation in the stomach (reddening, red stripes or crateriform raised areas). There was a non-statistically significant increasing trend for post-implantation loss which was most pronounced this dose.

Due to the mode of action of bicyclopyrone, tyrosine levels were measured in maternal animals. Bioanlaytical measurements in blood samples taken at 6 hours after the last application (day 27 *post coitum*) confirmed dose-dependent exposure of dams with bicyclopyrone. Blood concentrations of bicyclopyrone increased linearly with dose, although variability in the blood concentrations makes a quantitative dose relationship highly uncertain. Blood levels of tyrosine increased in a dose dependent manner and reached maximum values at 50 and 250 mg/kg/day.

Based upon the effects in this study, the maternal LOAEL is 250 mg/kg/day based upon macroscopic findings in the stomach wall of females and an increased incidence of post-implantation loss. The maternal NOAEL is 50 mg/kg/day.

Fetal effects are as follows:

At 10 mg/kg/day, there were no treatment-related fetal effects.

At 50 mg/kg/day bicyclopyrone, skeletal changes consisted of 27 pre-sacral vertebrae and malpositioned pelvic girdle.

At 250 mg/kg/day bicyclopyrone, fetal body weights were significantly decreased (↓11-13%). Skeletal variations noted in this dose group included increases in rib and cartilage variations, cervical vertebral ossification changes, pelvic displacement (reflecting the presence of an extra (27th) pre-sacral vertebra, and supernumerary ribs.

Based upon the effects in this study, the developmental LOAEL is 50 mg/kg/day based upon skeletal variations (27 prepelvic vertebra and malposistioned pelvic girdle). The developmental NOAEL is 10 mg/kg/day.

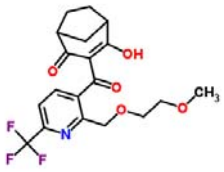
This study is classified as totally reliable (**acceptable/guideline)** and satisfies the registrants' need (OPPTS 870.3700b; OECD 414) for a developmental toxicity study in rabbits.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Technical, brown beige powder
Lot/Batch number:	SEZ3AP006/milled
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Stability confirmed (stored at a temperature < 30°C). Reanalysis date March 2011
Structures:	



Vehicle and/or positive control: The test substance was administered in 0.5% w/v aqueous carboxymethylcellulose (CMC), high viscosity.

Test Animals:	
Species	Rabbit
Strain	Himalayan
Age/weight at dosing	21-42 weeks/2279-3394 g
Source	Charles River Germany, Niederlassung Kisslegg, Stolzenseeweg 32-36, 88353 Kisslegg, Germany
Housing	Individually in stainless steel cages
Acclimatisation period	At least 5 days
Diet	Pelleted standard Kliba-Nafag 3418 rabbit maintenance diet (Provimi Kliba AG, 4303 Kaiseraugst, Switzerland) *ad libitum*
Water	Community tap water *ad libitum*
Environmental conditions	Temperature: 18±3°C Humidity: 30-70% Air changes: 10-15 changes/hour Photoperiod: 12 hours light/12 hours dark

Study Design and Methods:

In-life dates: Start: 30 March 2009 End: 19 May 2009

Mating procedure: After acclimatisation, females were placed in cages with sexually mature males (1:1) until copulation had been observed. After mating, the females were removed and placed in individual cages. The day of mating was designated day 0 *post coitum*.

Male rabbits from the same source and strain were used for the mating only. These male rabbits are in the possession of Harlan Laboratories Ltd. and were not considered part of the test system. The fertility of these males had been proven and was continuously monitored.

Animal assignment: Allocation procedure based on body weight, adjusted if necessary, so that a similar number of rabbits was allocated to each group on each day of mating and ensuring an acceptable distribution of males to which the females were mated.

Table 1: Animal numbers and treatment groups

Group	Test substance	Dose level (mg/kg/day)	Dose volume (mL/kg)	Number of females
1	Vehicle (CMC)	0	4	22
2	NOA4499280	10	4	22
3	NOA4499280	50	4	22
4	NOA4499280	250	4	22

Table was taken from page 20 of the study report

Dose selection rationale: The dose levels were selected based on a previous dose range finding toxicity study in Himalayan rabbits, Harlan Laboratories study B50523, using dose levels of 0, 10, 50 and 250 mg/kg/day.

Dosage preparation and analysis: The dose suspensions were prepared weekly. The test item was weighed into a glass beaker on a tared precision balance and approximately 80% of the vehicle added (w/v). Correction was not made for purity. After using a magnetic stirrer, the remaining vehicle was added and an ultra-turrax was used to prepare a homogeneous suspension. Each preparation was subdivided into aliquots. New aliquots were used for each day of the study. The dose preparations were stored in the refrigerator (5±3°C) until required for use.

Samples for determination of concentration, homogeneity and stability (7 days) of the dose formulations were taken on the first treatment day. During the last week of treatment, samples were taken to confirm concentration. The test item concentrations were determined by HPLC coupled to a UV/VIS detector and quantified with the area under the peak.

Concentration analysis results: Achieved concentrations were within 91.3-108.0% of nominal.

Homogeneity results: Percentage deviations were within the acceptable range of the overall mean for each concentration. Homogenous distribution was demonstrated because single results found did not deviate more than 3.2% (<15%) from the corresponding mean.

Stability: Based upon the results of stability analyses performed within the Harlan Laboratories Study B50523, dose suspensions were stable for at least 7 days when kept refrigerated.

The analytical data indicate that the test substance was homogeneous and stable in the suspensions and that the variation between the nominal and actual dosages to the animals was acceptable.

Dosage administration: The rabbits were dosed orally, by gavage, at a constant dose volume of 4 mL/kg according to daily individual body weights.

Homogeneity of the test item in the vehicle was maintained during the daily administration period using a magnetic stirrer.

Duration of dose administration: The rabbits were dosed on days 6-27 (inclusive) *post coitum.*

Observations:
Maternal observations: Animals were observed for mortality twice daily. Clinical signs were assessed daily for signs of reaction to the treatment and / or symptoms of ill health. Body weights were recorded daily from day 0 until day 28 *post coitum*. Food consumption was recorded at the following intervals: days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 21-24 and 24-28 *post coitum*.

On the last day of treatment (day 27 *post coitum*), blood samples (approximately 2 mL) were collected from the ear vein from all females each in groups 1 to 4. The samples were taken from each of the females 6 hours after administration. The blood samples were subdivided and prepared accordingly for plasma level determination of bicyclopyrone and L-tyrosine (using a sample preparation technique and a LC/MS/MS (liquid chromatography coupled with tandem mass spectrometric detection) method, formally validated according to GLP guidelines within Harlan Laboratories studies B95220 (NOA449280) and B95231 (Tyrosine)) and for haematology and clinical chemistry,

The following haematology parameters were determined: Erythrocyte count, mean corpuscular haemoglobin concentration, haemoglobin, haematocrit, mean corpuscular volume, red cell volume distribution width, mean corpuscular haemoglobin, haemoglobin concentration distribution width, total leukocyte count, differential leukocyte count and platelet count.

The following clinical biochemistry parameters were determined: Glucose, urea, creatinine, bilirubin, total cholesterol, total triglycerides, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl-transferase, creatine kinase, sodium, potassium, chloride, calcium, phosphorus, protein, total, electrophoresis and albumin/globulin ratio.

At the scheduled necropsy on day 28 *post coitum*, females were killed by an intravenous injection of sodium pentobarbital and the foetuses removed by Caesarean section. Any female killed during the study was subjected to macroscopic examination with emphasis on the uterus and its contents.

Post mortem examination, including gross macroscopic examination of all internal organs with emphasis on the uterus, uterine contents, position of foetuses in the uterus and the number of *corpora lutea*, was performed and the data recorded. The uteri (and contents) of all females with live foetuses were weighed during necropsy on day 28 *post coitum* to enable the calculation of the corrected body weight gain.

The liver weight of the dams was recorded.

Foetal observations: Foetuses were removed from the uterus, sexed, weighed individually, examined for gross external abnormalities, killed by a subcutaneous injection of sodium pentobarbital and allocated to one of the following procedures:

1. The foetuses were dissected; the organs examined and any abnormal findings were recorded. The sex of each foetus was noted.

2. After the skin had been removed, the cranium was examined for the degree of ossification.

3. From half of the foetuses the heads were separated from the trunks and fixed in Bouin's fixative. They were serially sectioned and examined (evaluation of the internal structures of the heads, including the eyes, brain, nasal passages and tongue) (J.G. Wilson, 1965). Descriptions of any abnormal findings were recorded. After examination, the sections were preserved in a solution of ethyl alcohol and glycerine (one head per container). From the other half of the foetuses the heads were not separated but processed and stained as described in the next paragraph.

4. From all foetuses the skin with the exception of over the paws and the dorsal-cervical fat pads were removed.

The trunks of the foetuses without heads and the foetuses with heads were processed through solutions of ethanol, glacial acetic with Alcian blue (for cartilage staining), potassium hydroxide with Alizarin red S (for clearing and staining ossified bone) and aqueous glycerin for preservation and storage (M. Inouye, 1976). The skeletons were examined and all abnormalities (malformations) and variations were recorded. The specimens were preserved individually in plastic vials.

Indices: The following indices were calculated from caesarean section records of animals in the study: Pre and post-implantation losses, embryonic and foetal deaths, live and dead foetuses, abnormal foetuses, foetal sex ratios and foetal body weights. For reproduction data, group mean values were calculated both on a litter basis and on a percentage per group basis. Mean foetal weights were calculated from the individual weights on a per litter basis.

Historical control data: Historical control data were provided for all reproductive and foetal findings observed in the current study. These historic control data were taken from 6 studies in the Himalayan rabbit, evaluated by similar foetal examination criteria, in this laboratory between 2006 and 2008.

Statistical analyses: The following statistical methods were used to analyse maternal, reproduction and skeletal examination data:
• Means and standard deviations of various data were calculated and included in the report.
• All statistical tests were two-sided.
• Statistical significance between groups was evaluated by Analysis of Variance (ANOVA). In the case where variances were non-homogeneous, appropriate transformations were applied (e.g. log, square root, or double arcsine) to stabilise the variances before the ANOVA. The Dunnett many-one t-test was then used to compare each group to control based on the error mean square in the ANOVA.
• Fisher's Exact-test was applied if the variables could be dichotomised without loss of information.
• For statistical tests on foetal data, comparisons were made between groups for number of foetuses affected and number of litters affected, for completeness. The litter was considered the proper unit of measurement for overall study evaluation.

RESULTS

Maternal toxicity:

Mortality and clinical signs: All female animals treated at 250 and 10 mg/kg bw/day and of the control group survived until scheduled necropsy. At 50 mg/kg bw/day, one female was found with a total abortion on day 28 *post coitum*, and was killed.
There were no test item-related clinical signs or symptoms noted at any dose level.

Body weight: There was no change in absolute body weight in maternal animals. The corrected body weight gains (corrected for gravid uterus weight) were -5.3%, -4.5%, and -4.5% in groups, 2, 3, and 4, compared to -5.1% in the control group, and thus similar in all groups. See tables 2 and 3.

Table 2: Intergroup comparison of absolute body weights (g) (selected time points)

	Dose level of bicyclopyrone (mg/kg/day)			
Study day	**0 (control)**	**10**	**50**	**250**
7	2830 ± 208.9	2789 ± 194.7	2785 ± 161.1	2802 ± 185.8
15	2880 ± 180.2	2836 ± 178.2	2827 ± 138.6	2836 ± 176.7
28	2977 ± 174.0	2947 ± 185.7	2944 ± 144.1	2952 ± 187.2

Data were taken from pages 41-42 of the study report

Table 3: Intergroup comparison of body weight gain (g) (selected time points)

	Dose level of bicyclopyrone (mg/kg/day)			
Study day	**0 (control)**	**10**	**50**	**250**
6-7	2.95 ± 13.6	-5.36 ± 18.6	-1.35 ± 18.0	-9.41 ± 16.5
6-15	52.1 ± 58.5	41.6 ± 52.7	40.3 ± 67.4	24.5 ± 52.6
6-28	149.9 ± 76.4	151.8 ± 106.7	157.9 ± 115.4	140.5 ± 117.2

Data were taken from pages 43-44 of the study report

Food consumption: No treatment-related effects were seen.

Bioanalytics: Due to the mode of action of bicyclopyrone, tyrosine levels were measured in maternal animals. Bioanlaytical measurements in blood samples taken at 6 hours after the last application (day 27 *post coitum*) confirmed dose-dependent exposure of dams with bicyclopyrone. Blood concentrations of bicyclopyrone increased linearly with dose, although variability in the blood concentrations makes a quantitative dose relationship highly uncertain. Blood levels of tyrosine reached saturation between the 50 and 250 mg/kg/day doses. See table 4.

Table 4: Mean Plasma Concentrations of bicyclopyrone and L-tyrosine

Dose Group (mg/kg/day)	Bicyclopyrone (µg/ml)	L-tyrosine (µg/ml)
0	<LLOQ	10.76 ± 2.52
10	0.385 ± 0.318	46.57 ± 15.34
50	10.97 ± 30.13	81.82 ± 24.07
250	97.12 ± 76.66	111.56 ± 25.53

Table was taken from page 20 of the study report
Values represent means ± standard deviations of plasma levels from pregnant dams. LLOQ=lower limit of quantification.

Haematology: The mean red cell distribution width (RDW) value was statistically significantly higher at 250 mg/kg/day (↑4%). This difference was considered not toxicologically significant.

Statistically significant decreases in mean haematocrit (HCT) values in the 10 and 250 mg/kg/day dose groups (both ↓5%), which were only minimally different from the control group value and without clear dose-dependency, were considered to be of incidental occurrence.

There were no other changes in hematological parameters in any dose group.

Clinical biochemistry: The only clinical chemical change was a modest in increase in plasma potassium levels at all three doses tested (↑11-16%). There were no other dose-related changes in clinical chemistry parameters in treated animals.

Sacrifice and pathology:
Maternal gross pathology: At 250 mg/kg/day body weight, 3 dams of 24 had necropsy findings suggesting irritation in the stomach (reddening, red stripes or crateriform raised areas), which was considered to be related to the treatment. No other test item-related findings were noted in any dam at scheduled necropsy. Other findings observed (e.g., increased or discoloured gall bladder, interrupted uterus horn or ovarian cyst) were of congenital origin or were isolated incidents and were therefore considered to be not test item-related. See table 5.

Table 5: Intergroup comparison of selected maternal macroscopic findings

	Dose level of bicyclopyrone (mg/kg/day)			
Finding	0 (control)	10	50	250
Stomach, crateriform raised area (s)	0	0	0	2
Stomach, red stripe	0	0	0	2
Stomach, reddining	0	0	0	1

Data were taken from page 57 of the study report

Liver weight: At all dose levels, the mean liver weight was similar to that of the control group.

Caesarean section data: There was no effect of treatment on embryonic or foetal deaths, or on the number of dead or live foetuses. There did however appear to be a non-statistically significant increasing trend for post-implantation loss which was most pronounced at 250 mg/kg/day (21.8% loss of implantation sites compared to 13.9% in controls) but also at 50

mg/kg/day (20.3% loss of implantation sites), which were considered to be treatment related. These increases were outside the historical control range. See table 6.

One dam (no. 53) in the 50 mg/kg/day dose group had a single resorption only. This animal was excluded from calculations.

Table 6: Summary of Caesarean section data

Observation	Dose level of bicyclopyrone (mg/kg bw/day)			
	0 (control)	10	50	250
Number of dams	21	21	19	21
Corpora lutea	165	165	147	162
Pre-implantation loss	23	16	14	15
% of *corpora lutea*	13.9	9.7	9.5	9.3
mean	1.1 ± 1.7	0.8 ± 1.3	0.7 ± 1.0	0.7 ± 1.1
Number of dams affected	10	8	9	8
Implantation sites	142	149	133	147
% of *corpora lutea*	86.1	90.3	90.5	90.7
mean	6.8 ± 1.7	7.1 ± 2.0	7.0 ± 2.3	7.0 ± 1.8
Post-implantation loss	19	20	27	32
% of implantation sites	13.4	13.4	20.3	21.8
mean	0.9 ± 1.0	1.0 ± 1.1	1.4 ± 1.5	1.5 ± 1.7
Number of dams affected	12	11	10	12
Implantation site scars	0	0	0	0
Embryonic / fetal deaths total	19	20	27	32
Embryonic resorptions	16	18	19	26
% of implantation sites	11.3	12.1	14.3	17.7
mean	0.8 ± 0.9	0.9 ± 1.1	1.0 ± 1.3	1.2 ± 1.6
Number of dams affected	10	10	8	11
Fetal resorptions	3	2	8	6
% of implantation sites	2.1	1.3	6.0	4.1
Total fetuses	123	129	106	115
Live fetuses	123	129	106	115
% of implantation sites	86.6	86.6	79.7	78.2
mean	5.9 ± 1.9	6.1 ± 2.0	5.6 ± 2.7	5.5 ± 2.2
Number of male fetuses	61	69	53	52
% of male fetuses	49.6	53.5	50.0	45.2
Number of female fetuses	62	60	53	63
% of female fetuses	50.4	46.5	50.0	54.8
Mean litter weight (g)	32.2 ± 2.6	31.5± 3.4	31.2 ± 2.4	28.4** ± 1.9 (↓12%)
Mean litter weight – males (g)	32.1 ± 2.9	31.4 ± 3.3	30.7 ± 2.7	27.9** ± 1.9 (↓13%)
Mean litter weight – females (g)	32.2 ± 2.8	31.4 ± 4.2	31.2 ± 2.3	28.5** ± 2.2 (↓11%)

Data were taken from pages 54-55 of the study report

** statistically significant difference from control at the 1% level (Dunnett test) based on pooled variance significance

Fetal observations: Sex ratios were not affected by treatment with the test item.

Calculated on litter basis, the mean body weight of fetuses was statistically significantly lower at the 250 mg/kg /day dose level when compared to controls which was considered to be related to the treatment with the test item. There was no test item-related effect on foetal body weights at 50 or 10 mg/kg/day. See tables 6.

Intergroup comparison of treatment-related findings in fetuses (litters) are presented in table 7. Based on litter evaluation, the incidence of abnormalities (malformations) was statistically significantly increased in all dose groups when compared to controls. There were, however, no external or visceral abnormalities observed in the control group, which is uncharacteristic.

Regarding visceral variations, At 10 and 50 mg/kg/day statistical differences from controls was the result of an increase in urogenital abnormalities. The unilateral absence of a kidney and ureter accounted for the majority of the urogenital abnormalities observed in all dose groups, but the incidence did not follow a dose-related pattern with increasing dose. In a subsequent study (R. Gerspach), the presence of unilateral missing kidney and ureter was observed in 2 of 88 maternal animals. In addition, unilateral missing or interrupted uterine horn was observed in 3 of 88 females in this study, as well as the subsequent study. This strongly supports that these observations reflect a genetic predisposition to this condition unrelated to exposure to any test material. Other abnormalities in these dose groups were isolated events and therefore considered not to be related to treatment.

Examination of sectioned fetal heads did not reveal any test item-related findings. One foetus in the 10 mg/kg/day dose group (with microphthalmia noted at external examination) with a supernumerary extraorbital chamber, small vitreous chamber, and multiple retinal folds, and one fetus in 250 mg/kg/day dose group with cleft palate and abnormalities in the nasopharynx and brain optic chiasma regions, were observed.

At 250 mg/kg/day, a statistically significant increase in the overall incidence of skeletal abnormalities was considered treatment-related. Cervical vertebral irregularities (mostly absence, fusions, malformation of vertebral body and/or arches of vertebrae 2 or 3 occurred with an increased incidence with 9 litters affected, versus one in the control group. In addition, there were increased incidences of abnormalities affecting rib1 and/or costal cartilage (short, interrupted, absent) in 16 foetuses in 7 litters, versus 2 in 2 litters in the control group. There were no significant differences in the incidence of skeletal abnormalities at 10 or 50 mg/kg body weight/day.

Heart findings (interventricular septum variations in the perimembranous region, including small septal defects, diverticulum or abnormal surface appearance) occurred in statistically significant higher incidences (a total of 19 litters versus 5 in the control group). These changes reflect minor differences in the appearance of the septum indicating a slight delay in the closure of the membraneous portion of the interventricular septum in animals that are examined 24-48 hours prior to normal birth. A statistically higher foetal and litter incidence of an abnormal surface appearance was observed at 10 mg/kg/day and above, compared to controls. A subsequent study (R. Gerspach) revealed that the background incidence of these variations can be substantial, reflecting the stage of development at which these foetuses are examined. Syngenta subsequently submitted a report (MRID 49383701) characterizing why these heart variations are of low concern (see reviewer comments). After review of this weight of evidence, these changes are considered treatment related only at 250 mg/kg/day.

Treatment-related increases in the litter incidence of skeletal variations were observed at 250 and 50 mg/kg /day. At 250 mg/kg/day, incomplete ossification of cervical vertebrae (body/arch, odontoid process), cervical vertebra 2 supernumerary ossification, pelvic girdle malpositioned caudal (also known categorized as the presence of 27 pre-sacral vertebrae), and costal cartilage asymmetrically aligned at the sternum were significantly increased. At 50 mg/kg/day, statistically significant increases in pelvic girdle malpositioned and asymmetrically aligned costal cartilage were noted.

At 250 mg/kg body weight/day, statistically significant increases in the litter incidence of supernumerary ribs, delayed ossification of the pubis, incomplete ossification of the 5th medial phalange, and an additional ossification centre in the femur were observed

Other variations were not statistically significant or without dose-dependency and/or were within the range of historical reference data, and were therefore considered to be incidental.

Examination of the cartilage for additional variations revealed statistically significant increases in the litter incidences of costal cartilage variations at 250 and 50 mg/kg body weight/day.

Table 7: Intergroup comparison of treatment-related findings in fetuses (litters)

Finding	Dose level of bicyclopyrone (mg/kg/day)			
	0	10	50	250
Number of foetuses (litters) examined	123 (21)	129 (21)	106 (19)	115 (21)
Visceral abnormalities				
Heart muscular interventricular septal defect	0 (0)	0 (0)	0 (0)	3 (3)
Urogenital structure(s) absent or misshapen/ malpositioned (total)	0 (0)	3 (3)	4* (4)*	5* (5)*
Kidney and ureter absent	0 (0)	3 (3)	2 (2)	4 (4)
Ovary/uterine horn or testis/vas deferens absent or misshapen/malpositioned	0 (0)	3 (3)	3 (3)	5* (5)*
Visceral variations				
Heart interventricular septum variations (total)	6 (5)	17* (8)	21** (10)	42** (19)**
Heart Perimembraneous region: Small septal defect	3 (3)	7 (4)	3 (3)	18** (13)**
Heart Perimembraneous region: Diverticulum	1 (1)	0 (0)	3 (2)	8* (8)*
Heart Perimembraneous region: Abnormal surface appearance	2 (2)	10* (7)*	14** (8)*	15** (10)**
Skeletal abnormalities				
Cervical vertebral irregularities(total)	1 (1)	1 (1)	4 (3)	19** (9)**
Vertebrae 2, 3 body/odontoid process/arch absent /misshapen / fused / not fused dorsal / small / split /supernumerary	1 (1)	1 (1)	4 (3)	19** (9)**
Vertebrae 4, 5, 6 body/arch absent / fused /interrupted / not fused dorsal / small / split	0 (0)	0 (0)	1 (1)	5* (3)
Ribs and/or costal cartilage irregularities (total)	2 (2)	0 (0)	4 (4)	16** (7)
Thoracic rib/costal cartilage 1, 2 absent / branched /interrupted / malpositioned / partially duplicated /short	1 (1)	0 (0)	3 (3)	12** (7)*
Other thoracic ribs/costal cartilages absent /	0 (0)	0 (0)	1 (1)	6* (2)

fused /interrupted / misshapen / supernumerary				
Skeletal variations				
Cervical vertebra incompletely ossified (includes isolated ossification site) (total)	2 (2)	0 (0)	1 (1)	18** (10)**
Vertebrae1- 3 body/arch incompletely ossified (includes unilateral ossification), non-ossified or isolated ossification site	1 (1)	0 (0)	1 (1)	8* (6)*
Vertebra 2 odontoid process non-ossified	0 (0)	0 (0)	0 (0)	13** (8)**
Pelvic girdle malpositioned caudal (total)	3 (2)	3 (3)	22** (11)**	79** (21)**
27 prepelvic vertebrae (total)	3 (2)	3 (3)	21** (10)**	79** (21)**
Costal cartilages asymmetrically aligned at sternum	1 (1)	3 (3)	6* (6)*	10** (9)**
Costal cartilage 8 (false rib) connected to sternum	0 (0)	0 (0)	0 (0)	4 (4)
* significant at 5% level, ** significant at 1% level (Fischer's Exact Test)				

Data were taken from pages 63-71 of the study report

Table 8. Historical Control Data- Oral Gavage sets (Chosen for similar experimental conditions)

Study Reference	06/05	07/03	07/04
Interventricular septum, Defect perimembraneous regions	1/18 (6%)	3/28 (11%)	1/17 (6%)

Page 204 of MRID 47841998 and page 192 of 47841999

INVESTIGATOR'S CONCLUSIONS: Based on macroscopic findings in the stomach wall of females given 250 mg/kg/day, the maternal no-observed-effect level (NOAEL) in this study was 50 mg/kg/day. The no observed adverse effect level (NOAEL) for foetal effects was 10 mg/kg/day.

REVIEWER'S COMMENTS: Syngenta performed a follow up study to the current developmental Himalayan rabbit study (MRID 47841999) with the intention of increasing the likelihood of establishing a developmental NOAEL as there are a myriad of foetal effects in both studies. The intent was also for the two studies to be considered together.

When looking at the effects in fetuses, there appeared to be a treatment related effect in the heart described as "Interventricular Septal Variations," which was previously the basis for setting the LOAEL at the lowest dose tested. However, after examination of the historical control data in both the 47841998 and the 47841999 studies, there appears to be no dose response for this effect. Furthermore the "total variations" were not reported in the original Tier II summary for study 47841999 and are depicted along with the results from this study in the following table below:

Bicyclopyrone-mediated fetal heart effects in Himalayan Rabbits - Interventricular Variations (per litter basis)					
Dose Levels (mg/kg/day)	**0**	**1**	**10**	**50**	**250**
47841998	5/21	NA	8/21	10/19	19/21
47841999	15/22	6/20	10/20	NA	13/18
Total	20/43	6/20	18/41	10/19	32/39
Percent	47%	30%	44%	50%	82%

Data are the sum of sets from the 47841998 (page 65) and 47841999 (page 75)

In a subsequent report submitted by Syngenta (MRID 49383701), changes to the micro-dissection method used for looking at changes to the fetal heart were described. Specifically Syngenta revised the Stuckhardt and Poppe method which allowed for better viewing of the above discussed Interventricular Septal variations, which were later in part determined to be an artifact of the revised dissection technique.

The report also described how variations such as those observed in the Himalayan rabbit foetal heart are known to be spontaneous and eventually resolve themselves. Furthermore characterization was provided stating that this species is not scientifically the best model for assessing developmental toxicity. Considered together, these data suggest there is a treatment related increase in these variations is only present at 250 mg/kg/day and the developmental LOAEL will now be set for the observed skeletal effects at 50 mg/kg/day based upon increased incidences of non-ossified vertebra, 27 prepelvic vertebra and malposistioned pelvic girdle. The NOAEL will be set at 10 mg/kg/day.

Based upon the effects in this study, the maternal LOAEL is 250 mg/kg/day based upon macroscopic findings in the stomach wall of females and an increased incidence of post-implantation loss. The maternal NOAEL is 50 mg/kg/day.

Based upon the effects in this study, the developmental LOAEL is 50 mg/kg/day based upon skeletal variations (27 prepelvic vertebra and malposistioned pelvic girdle). The developmental NOAEL is 10 mg/kg/day.

This study is classified as totally reliable (**acceptable/guideline)** and satisfies the registrants' need (OPPTS 870.3700b; OECD 414) for a developmental toxicity study in rabbits. EPA, PMRA (Canada), APVMA/OCS (Australia) agree on the classification, but not the regulatory decision for this study. AMPVA considers the increased resorptions to be fetal as opposed to maternal effects.

References:

J.G. Wilson: In: Teratology: Principles and Techniques. Eds., J.G. Wilson and J. Warkany, University of Chicago Press, pp. 265-277 (1965)
Modification of M. Inouye: Differential staining of cartilage and bone in foetal mouse skeleton by Alcian blue and Alizarin red-S. Congenital Anomalies 16, pp. 171-173 (1976)
R. Gerspach: NOA449280 - Prenatal Developmental Toxicity Study in the Himalayan Rabbit (not yet published)

(Whitlow S, 2012)

Appendix

STUDY TYPE: Dose Range-finding Prenatal Developmental Study (rabbit). Not conducted to any specific regulatory guidelines but based on OECD 414 (2001): OPPTS 870.3700 (1998): JMAFF 12 NohSan No. 8147 (2000)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Whitlow S, 2012. NOA449280: Dose range finding prenatal developmental toxicity study in the Himalayan rabbit. Harlan Laboratories Ltd., (former RCC Ltd.), Wölferstrasse 4, 4414 Füllinsdorf, Switzerland. Laboratory Report No. B50523, 19 September 2012. Unpublished. Syngenta File No. NOA449280_11298 MRID 47841997.

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

Not conducted to any specific regulatory guidelines.

EXECUTIVE SUMMARY

The purpose of this study was to assess the effects of bicyclopyrone (purity 94.5%) on the pregnant female and the embryonic and foetal development when administered orally, by gavage, once daily to mated young adult female Himalayan rabbits from day 5 through to day 27 *post coitum*. The results of this study were used to establish suitable dose levels for a subsequent main prenatal developmental toxicity study in the rabbit.

Each group consisted of 10 mated female rabbits. Bicyclopyrone was administered at dose levels of: 0 (vehicle control), 10, 50, 250 mg/kg body weight/day. A standard dose volume of 4 mL/kg body weight with a daily adjustment to the actual body weight was used. Control animals were dosed with the vehicle alone (0.5% w/v aqueous CMC high viscosity).

All surviving females were killed on day 28 *post coitum* and the foetuses were removed by Caesarean section. Examination of dams and foetuses was performed in accordance with international recommendations.

All female animals survived until scheduled necropsy. No clinical signs or ophthalmoscopic effects were noted in any dams. There was no treatment related effect on body weight or food consumption.

At 250 mg/kg/day, the post-implantation loss was statistically significantly increased with 37.1% of implantation sites (37.1% embryonic resorptions). Thus, the mean number of total foetuses was statistically significantly decreased to 62.9% of implantation sites. At 10 and 50 mg/kg/day, pre-implantation loss, implantation rate, post implantation loss, and the number of living foetuses, respectively, was considered to be not influenced by treatment with the test item.

At necropsy, one dam at 10, 50, and 250 mg/kg/day were noted with crateriform elevations or depressions with increasing diameters at the pylorus area that were interpreted as beginning ulcerations. All other macroscopic findings were considered to be within the range of normal background alterations.

Based upon the results of this study, the maternal LOAEL is 250 mg/kg/day based upon a statistically significant increase in post-implantation loss, and an increase in resorptions. The maternal NOAEL is 50 mg/kg/day.

There were no effects on foetal sex ratios or mean foetal body weight. External examination of foetuses did not reveal any test item-related findings. Treatment-related increases in the overall incidence of visceral abnormalities were observed at 250 and 50 mg/kg/day. At 250 mg/kg/day, the majority of affected foetuses exhibited interventricular septal defect (8 foetuses in 5 litters) and at 50 mg/kg/day, 3 of the 4 foetuses with abnormalities exhibited interventricular septal defects.

Skeletal abnormalities were dose-dependently noted at 50 and 250 mg/kg/day as multiple cervical vertebrae misshapen, supernumerary or fused, cervicothoracic vertebrae fused, thoracic vertebra absent, thoracolumbar vertebrae fused, scoliosis, and rib interrupted or short, and were considered test item-related.

The bone variations were noted with dose-dependency and mostly outside the range of historical reference data at 250 mg/kg/day. These were considered likely test item-related. Skeletal examination (stage of development) of foetuses revealed a mostly dose-dependently increased ossification of sternebra 5, but incompletely or non-ossified sternebra 1, dose dependently increased supernumerary ribs, and decreasing additional ossifications of humerus, femur and tibia bones. These findings were considered likely test item-related.

At 250 mg/kg/day, one foetus was noted with severely dilated lateral ventricles of the brain, bilateral (internal hydrocephaly) that was considered test item-related.

Cartilage abnormalities were dose-dependently noted at 50 mg/kg/day (1 foetus) and at 250 mg/kg/day (11 foetuses in 7 litters) and were considered test item-related. Common cartilage variations were dose-dependent and at 250 mg/kg/day outside the historical control data. Corresponding to the skeletal examination, the incidence was also considered likely to reveal test item related effects.

Based upon the effects in this study, the developmental LOAEL is 10 mg/kg/day based upon an increase in the incidence of skeletal variations. The developmental NOAEL was not observed.

This study is classified **acceptable/non-guideline** and satisfies the intent of the guideline requirement (OPPTS 870.3700b; OECD 414) for a range-finding developmental toxicity study in rabbits.

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) Date: 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) Date: 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Prenatal Developmental Study (rabbit). OECD 414 (2001): OPPTS 870.3700 (1998): EC 2004/73 B31 (B31): MAFF 12NohSan No. 8147 (2000)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Whitlow S, 2012. NOA449280: Prenatal developmental toxicity study in the Himalayan rabbit. Harlan Laboratories Ltd., Wölferstrasse 4, 4414 Füllinsdorf, Switzerland. Laboratory Report No. C91501, 19 September 2012. Unpublished. Syngenta File No. NOA449280/11300. MRID 47841999

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a developmental toxicity study in Himalayan rabbits (MRID #47841999), bicyclopyrone (NOA449280, purity 94.5%) was administered to pregnant Himalayan rabbits. The effects of bicyclopyrone on the development of the embryos and fetuses consequent to exposure of the female to the test item from day 6 *post coitum* (implantation) to day 27 *post coitum* (the day prior to Caesarean section) were determined.

Each group consisted of 22 mated young adult female rabbits. Bicyclopyrone was administered at dose levels of: 0 (vehicle control), 1, 10 or 250 mg/kg/day. A standard dose volume of 4 mL/kg body weight with a daily adjustment to the actual body weight was used. Control animals were dosed with the vehicle alone (0.5% carboxymethyl cellulose high viscosity). At scheduled sacrifice on day 28 *post coitum*, the females were killed and the fetuses were removed by Caesarean section. Examination of dams and fetuses was performed in accordance with international recommendations.

The maternal effects are as follows:

All female animals treated at 1 and 10 mg/kg/day and of the control group survived until scheduled necropsy. There were no effects in these dose groups.

At 250 mg/kg/day two dams were killed on day 22 *post coitum* for ethical reason. Before, both animals showed general weak condition, decreased activity and prostrate position. Post-implantation loss was statistically significantly increased (32% of implantation sites), due to an increased number of embryonic resorptions, and the number of fetuses at Caesarean section was consequently reduced. The two dams, which were sacrificed prematurely, had macroscopic findings, at examination *post mortem*, indicative of irritations in the stomach (red and dark red foci).

Based upon the mode of action of bicyclopyrone, bioanalytical measurements in blood samples taken on the last day of treatment (day 27 *post coitum*) confirmed the dose-dependent exposure of dams with bicyclopyrone and indicated the dose-dependent increase of Tyrosine levels.

Based upon the results in this study, the maternal LOAEL is 250 mg/kg/day based upon signs of maternal toxicity including two early deaths, signs of stomach irritation, and an increase incidence of post-implantation loss. The maternal NOAEL is 10 mg/kg/day.

Fetal effects are as follows:

There was no effect on fetal sex ratios. The overall incidence of abnormalities at 1 and 10 mg/kg/day was not increased when compared to controls. The only change at 10 mg/kg/day was a low incidence of costal cartilage not reaching the sternum which was not considered adverse.

At 250 mg/kg/day bicyclopyrone, the mean body weight of fetuses was statistically significantly decreased (↓12%). At external examination, jaw and/or cleft palate was observed in 3 fetuses of 3 litters; visceral examination revealed heart muscular and/or perimembraneous interventricular septal defects and misshapen internal musculature in the septum. Visceral variations attributed to treatment included increased incidences of malpositioned esophagus and aortic arch supernumerary branch. Urogenital findings (absent kidney and ureter, malpositioned kidney and dilated ureter, absent uterine horn and misshapen ovary) were noted in all treated groups.

An overall increase in skeletal abnormalities was observed. These effects were primarily cervical vertebral irregularities (mostly absence, fusions, malformation of vertebral body and/or arches of vertebrae 2 or 3) and abnormalities affecting ribs 1 or 2 and/or costal cartilage (short, interrupted, fused). The incidence of bone and cartilage variations was also increased in this dose group, consisting of cervical vertebrae structural variations (absent transverse foramen, supernumerary site ventral) and ossification irregularities (incomplete or no ossification, isolated ossification site) and caudal displacement of the pelvic girdle.

Increases in full supernumerary thoracolumbar ribs and incompletely ossified right and left pubis were considered related to treatment. Cartilage variations included increased incidences of right and/or left costal cartilage 1 not reaching sternum; decreased incidence of right and/or

left costal cartilage 7 not reaching sternum; and increased incidence of supernumerary costal cartilages.

Based upon the effects of this study, the developmental LOAEL is 250 mg/kg/day based upon multiple external, visceral and skeletal abnormalities, and multiple visceral skeletal, bone and cartilage variations. The developmental NOAEL is 10 mg/kg/day.

This study is classified as totally reliable (**acceptable/guideline)** and satisfies the registrants' need (OPPTS 870.3700b; OECD 414) for a developmental toxicity study in rabbits.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS
Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Technical, brown beige powder
Lot/Batch number:	SEZ3AP006/milled
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Stability confirmed (stored at a temperature < 30°C). Reanalysis date March 2011
Structures:	

Vehicle and/or positive control: The test substance was administered in 0.5% w/v aqueous carboxymethylcellulose (CMC), high viscosity.

Test Animals:

Species	Rabbit
Strain	Himalayan (SPF): Crl:CHBB(HM)
Age/weight at day 0 post coitum	At least 17 weeks/2270-3727 g
Source	Charles River Germany, Niederlassung Kisslegg, Stolzenseeweg 32-36, 88353 Kisslegg, Germany
Housing	Individually in stainless steel cages
Acclimatisation period	At least 5 days
Diet	Pelleted standard Kliba-Nafag 3418 rabbit maintenance diet (Provimi Kliba AG, 4303 Kaiseraugst, Switzerland) *ad libitum*
Water	Community tap water *ad libitum*
Environmental conditions	Temperature: 18±3°C Humidity: 30-70% Air changes: 10-15 changes/hour Photoperiod: 12 hours light/12 hours dark

Study Design and Methods:
In-life dates: Start (start of dosing): 06 September 2010 End: 18 October 2010

Mating procedure: After acclimatisation, females were placed in cages with sexually mature males (1:1) until copulation had been observed. After mating, the females were removed and placed in individual cages. The day of mating was designated day 0 *post coitum*.

Male rabbits from the same source and strain were used for the mating only. These male rabbits are in the possession of Harlan Laboratories Ltd. and were not considered part of the test system. The fertility of these males had been proven and was continuously monitored.

Animal assignment: Allocation procedure based on body weight, adjusted if necessary, so that a similar number of rabbits was allocated to each group on each day of mating and ensuring an acceptable distribution of males to which the females were mated.

Table 1: Animal numbers and treatment groups

Group	Test substance	Dose level (mg/kg/day)	Dose volume (mL/kg)	Number of females
1	Vehicle (CMC)	0	4	22
2	NOA4499280	1	4	22
3	NOA4499280	10	4	22
4	NOA4499280	250	4	22

Table was taken from page 20 of the study report

Dose selection rationale: The dose levels were selected based on a previous developmental toxicity study in Himalayan rabbits, Harlan Laboratories study C41898, using dose levels of 10 mg/kg/day and above, in which a NOEL could not be defined (MRID #47841999). Based on this previous study, dose levels of 1, 10 and 250 mg/kg body weight/day were selected for this study. A dose of 250 mg/kg body weight/day was expected to reproduce findings observed in the initial study. Dose levels of 1 and 10 mg/kg body weight/day were selected to help define a no-observed-effect level for foetal development. Also see the Appendix for the dose range-finding study in Himalayan rabbits.

Dosage preparation and analysis: The dose suspensions were prepared weekly. Bicyclopyrone was weighed into a plastic tray on a tared precision balance, transferred to a glass beaker and approximately 80% of the vehicle was added (w/v). Correction was not made for purity. Using a magnetic stirrer and an ultra-turrax, a homogeneous suspension was prepared, after which the vehicle was added to the final volume under continued stirring. Each preparation was subdivided into aliquots. New aliquots were used for each day of the study. The dose preparations were stored in the refrigerator until required for use. Homogeneity of the test item in the vehicle was maintained during the daily administration period using a magnetic stirrer.

Samples for determination of concentration, homogeneity and stability (7 days) of the dose formulations were taken from the first dosing mix. During the last week of treatment, samples were taken to confirm concentration. The test item concentrations were determined by HPLC coupled to a UV detector.
Concentration analysis results: Achieved concentrations were within 90.8-101.7% of nominal.

Homogeneity results: Percentage deviations were within the acceptable range of the overall mean for each concentration. Homogenous distribution was demonstrated because single results found did not deviate more than 0.9% (<15%) from the corresponding mean.
Stability: Based upon the results of stability analyses performed within the Harlan Laboratories Study C41898 dose formulations were stable for at least one week.

The analytical data indicate that the test substance was homogeneous and stable in the suspensions and that the variation between the nominal and actual dosages to the animals was acceptable.

Dosage administration: The rabbits were dosed orally, by gavage, at a constant dose volume of 4 mL/kg according to daily individual body weights.

Homogeneity of the test item in the vehicle was maintained during the daily administration period using a magnetic stirrer.

Duration of dose administration: The rabbits were dosed on days 6-27 (inclusive) *post coitum*.

Observations:
Maternal observations: Animals were observed for viability / mortality twice daily. Clinical signs were assessed daily for signs of reaction to the treatment and / or symptoms of ill health. Body weights were recorded daily from day 0 until day 28 *post coitum*. Food consumption was recorded at the following intervals: days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 21-24 and 24-28 *post coitum*.

On the last day of treatment (day 27 *post coitum*), blood samples (approximately 2 mL) were collected from the ear vein from all females each in groups 1 to 4. The samples were taken from each of the females 1 hour and 6 hours after administration. The samples were analysed for bicyclopyrone (1 hour and 6 hours after administration) and for Tyrosine (6 hours after administration) levels using a sample preparation technique and a LC/MS/MS (liquid chromatography coupled with tandem mass spectrometric detection) method, formally validated according to GLP guidelines within Harlan Laboratories studies B95220 (NOA449280) and B95231 (Tyrosine).

At the scheduled necropsy on day 28 *post coitum*, females were killed by an intravenous injection of sodium pentobarbital and the foetuses removed by Caesarean section. Any female killed during the study was subjected to macroscopic examination with emphasis on the uterus and its contents.

Post mortem examination, including gross macroscopic examination of all internal organs with emphasis on the uterus, uterine contents, position of foetuses in the uterus and the number of *corpora lutea*, was performed and the data recorded. The uteri (and contents) of all females with live foetuses were weighed during necropsy on day 28 *post coitum* to enable the calculation of the corrected body weight gain.

Foetal observations: Foetuses were removed from the uterus, sexed, weighed individually, examined for gross external abnormalities, killed by a subcutaneous injection of sodium pentobarbital and allocated to one of the following procedures:

1. After the skin had been removed, a more detailed external examination of the head was performed with emphasis on the eyes, sutures, fontanelles and skull bones.

2. The foetuses were dissected, the organs examined and any abnormal findings were recorded. The sex of each foetus was noted. These examinations on foetuses were performed without the examiners' knowledge of the dose group.

3. From half of the foetuses the heads were separated from the trunks and fixed in Bouin's fixative. They were serially sectioned and examined (evaluation of the internal structures of the heads, including the eyes, brain, nasal passages and tongue) (Wilson, 1965) Descriptions of any abnormal findings were recorded. After examination, the sections were preserved in a solution of ethyl alcohol and glycerine (one head per container). From the other half of the foetuses the heads were not separated but processed and stained as described in the next paragraph.

4. From all foetuses the skin, with the exception of over the paws, was removed. In addition, the dorsal-cervical and interscapular fat pads were removed.

The trunks of the foetuses without heads and the foetuses with heads were processed through solutions of ethanol, glacial acetic with Alcian blue (for cartilage staining), potassium hydroxide with Alizarin red S (for clearing and staining ossified bone) and aqueous glycerine for preservation and storage (Inouye, 1976). The skeletons were examined and all abnormal findings and variations were recorded.

Indices: The following indices were calculated from caesarean section records of animals in the study: Pre and post-implantation losses, embryonic and foetal deaths, live and dead foetuses, abnormal foetuses, foetal sex ratios and foetal body weights. For reproduction data, group mean values were calculated both on a litter basis and on a percentage per group basis. Mean foetal weights were calculated from the individual weights on a per litter basis.

Historical control data: Historical control data were provided for all reproductive and foetal findings observed in the current study. These historic control data were taken from 12 studies in the Himalayan rabbit, evaluated by similar foetal examination criteria, in this laboratory between 2006 and 2010.

Statistical analyses: The following statistical methods were used to analyse maternal, reproduction and skeletal examination data:

• Means and standard deviations of various data were calculated.
• All statistical tests were two-sided.
• Statistical significance between groups was evaluated by Analysis of Variance (ANOVA). In the case where variances were non-homogeneous, appropriate transformations were applied (e.g. log, square root, or double arcsine) to stabilise the variances before the ANOVA. The Dunnett many-one t-test was then used to compare each group to control based on the error mean square in the ANOVA.
• Fisher's Exact-test was applied if the variables could be dichotomised without loss of information.
• For statistical tests on foetal data, comparisons were made between groups for number of foetuses affected and number of litters affected, for completeness. The litter was considered the proper unit of measurement for overall study evaluation.

RESULTS

Maternal toxicity:

Mortality and clinical signs: In the high dose group (250 mg/kg/day), two dams were killed on day 22 *post coitum* as they were found with decreased activity, prostrate position and general weak condition. All female animals treated at 10 and 1 mg/kg/day and of the control group survived until scheduled necropsy.

There were no test item-related clinical signs or symptoms noted in animals at 10 or 1 mg/kg body weight. Other observations (hair loss, scabs or transient diarrhoea) in individual dams of groups 1, 3, and 4 were considered to be of incidental nature.

Body weight: There were no statistically significant differences in absolute body weights or body weight gains in any dose group. However, cumulative body weight gain in dams given 250 mg/kg/day was consistently lower than controls throughout most of the treatment period. See tables 2 and 3.

Table 2: Intergroup comparison of absolute body weights (g) (selected time points)

	Dose level of bicyclopyrone (mg/kg/day)			
Study day	**0 (control)**	**1**	**10**	**250**
7	2867 ± 303	2875 ± 358	2881 ± 329	2813 ± 258
15	2977 ± 285	3002 ± 272	2992 ± 306	2887 ± 244
28	3114 ± 282	3077 ± 240	3075 ± 211	2973 ± 226

Data were taken from pages 50-52 of the study report

Table 3: Intergroup comparison of body weight gain (g) (selected time points)

	Dose level of bicyclopyrone (mg/kg/day)			
Study day	**0 (control)**	**1**	**10**	**250**
7	4 ± 14	4 ± 15	-2 ± 18	-4 ± 27
15	57 ± 59	67 ± 81	54 ± 48	29 ± 47
28	193 ± 149	142 ± 165	136 ± 147	115 ± 92

Data were taken from pages 54-56 of the study report

Food consumption: No treatment-related effects were seen.

Bioanalytics: Based upon the mode of action of bicyclopyrone, bioanlaytical measurements in blood samples taken at 6 hours after the last application (day 27 *post coitum*) confirmed the dose-dependent exposure of dams with bicyclopyrone and indicated the dose-dependent increase of Tyrosine levels. See table 4.

Table 4: Mean Plasma Concentrations of bicyclopyrone and L-tyrosine

Dose Group (mg/kg/day)	Bicyclopyrone (µg/ml)	L-tyrosine (µg/ml)
1-hr. Post Dosing		
0	<LLOQ	ND
1	0.896 ± 0.44	ND
50	10.89 ± 7.01	ND
250	227.00 ± 80.19	ND
6-hr. Post Dosing		
0	<LLOQ	10.28 ± 1.75
1	0.294 ± 0.44	26.27 ± 11.19
50	2.18 ± 5.74	57.56 ± 23.35
250	104.68 ± 104.71	111.66 ± 28.66

Table was taken from page 30 of the study report
Values represent means ± standard deviations of plasma levels from pregnant dams. LLOQ=lower limit of quantification.

Sacrifice and pathology:

Maternal gross pathology: At 250 mg/kg/day, the two dams which were killed prematurely had findings indicative of irritations in the stomach (red or dark red foci), which were considered to be related to the treatment with the test item. See table 5.

No other test item-related findings were noted in any dam at scheduled necropsy. One dam each at 10 and 250 mg/kg bw/day had a unilateral missing kidney with accompanying or interrupted uterine horn.

Table 5: Intergroup comparison of selected maternal macroscopic findings

	Dose level of bicyclopyrone (mg/kg/day)			
Finding	**0 (control)**	**1**	**5**	**250**
Dark Red Foci (animals no. 70 and 80)	0	0	0	2

Data were taken from pages 372 to 383 of the study report

Caesarean section data: At 250 mg/kg/day, post-implantation loss was statistically significantly increased (32% of implantation sites, compared to 7.5% in the control group), due to an increased number of embryonic resorptions. Consequently, the number of foetuses at Caesarean section was smaller than in the control group.

There were no effects on the relevant reproduction parameters (post-implantation loss, embryonic or fetal deaths, number of dead or live foetuses) at 1 or 10 mg/kg/day.

One dam in the 1 mg/kg/day dose group and two dams at 10 mg/kg/day had resorptions only. These animals were excluded from calculations. See table 6.

Table 6: Summary of Caesarean section data

Observation	Dose level of bicyclopyrone (mg/kg bw/day)			
	0 (control)	**1**	**10**	**250**
Number of dams	22	20	20	18
Corpora lutea	174	147	145	145

Pre-implantation loss	14	25	14	20
% of *corpora lutea*	8.0	17.0	9.7	13.8
mean	0.6 ± 0.7	1.3 ± 1.8	0.7 ± 1.0	1.1 ± 1.4
Number of dams affected	11	9	8	9
Implantation sites	160	122	131	125
% of *corpora lutea*	92.0	83.0	90.3	86.2
mean	7.3 ± 2.4	6.1 ± 2.4	6.6 ± 1.9	6.9 ± 2.5
Post-implantation loss	12	16	17	40
% of implantation sites	7.5	13.1	13.0	32.0
mean	0.5 ± 0.7	0.8 ± 1.0	0.9 ± 1.2	2.2** ± 1.6 (↑340%)
Number of dams affected	10	10	9	15
Implantation site scars	0	0	0	0
Embryonic / fetal deaths total	12	16	17	40
Embryonic resorptions	7	10	13	34
% of implantation sites	4.4	8.2	9.9	27.2
mean	0.3 ± 0.5	0.5 ± 1.8	0.7 ± 1.0	1.9** ± 1.5 (↑533%)
Number of dams affected	7	8	8	14
Fetal resorptions	5	6	4	6
% of implantation sites	3.1	4.9	3.1	4.8
Total fetuses	148	106	114	85
Live fetuses	148	106	114	85
% of implantation sites	92.5	86.9	87.0	68.0
mean	6.7 ± 2.2	5.3 ± 1.8	5.7 ± 1.9	4.7*± 2.4 (↓30%)
Number of male fetuses	83	45	56	38
% of male fetuses	56.1	42.5	49.1	44.7
Mean	3.8 ± 2.0	2.3* ± 1.6	2.8 ± 1.5	2.1** ± 1.5 (↓45%)
Number of female fetuses	65	61	58	47
% of female fetuses	43.9	57.5	50.9	55.3
Mean	3.0	3.1*	2.9	2.6** (↓13%)
Mean litter weight (g)	31.2 ± 3.7	31.2 ± 5.2	32.1 ± 3.2	27.5* ± 3.2 (↓12%)
Mean litter weight – males (g)	31.0 ± 3.1	31.1 ± 4.8	32.6 ± 3.2	27.2* ± 4.1 (↓12.3%)
Mean litter weight – females (g)	30.7 ± 4.0	31.2 ± 5.2	31.6± 3.2	27.3* ± 3.9 (↓11.1%)

Data were taken from pages 61-62 of the study report

* statistically significant difference from control at the 5% level (Dunnett test) based on pooled variance significance

** statistically significant difference from control at the 1% level (Dunnett test) based on pooled variance significance

Fetal observations: Sex ratios were not affected by treatment with the test item.

Calculated on litter basis, the mean body weight of fetuses (males 27.2 g, females 27.3 g, and combined 27.5 g, versus 31.0 g, 30.7 g, and 31.2 g in the control group, respectively) was statistically significantly lower at the 250 mg/kg body weight dose level, which was

considered to be related to the treatment with the test item. There was no test item-related effect on fetal body weights at the 10 mg/kg or 1 mg/kg body weight dose levels. See table 6.

Treatment-relating findings in foetuses for all doses are presented in table 7. Based on litter evaluation, a statistically significant treatment-related increase in the overall incidence of external and visceral abnormalities (malformations) was observed at 250 mg/kg/day. Externally, jaw and/or palate cleft occurred in 3 foetuses from 3 separate litters (that was confirmed upon dissection of heads using the Wilson slice technique).

An increased incidence of heart muscular and/or perimembraneous interventricular septal defect was noted in 6 foetuses from 5 litters at visceral examination in this dose group. In addition, misshapen internal musculature in the septum associated with these septal defects was observed in 3 foetuses from 3 litters. These values were above the range of the historical control group reference data and the registrant submitted a supplemental report discussing this variation (see reviewer comments).

Urogenital findings (absent kidney and ureter, malpositioned kidney and dilated ureter, absent uterine horn and misshapen ovary) were noted in all treated groups. However, the presence of two dams with absent kidney in the current study and with missing uterus horn in this study indicated an underlying genetic cause rather than an effect of the test item. The overall incidence of abnormalities at 1 and 10 mg/kg/day was not increased when compared to controls.

At 250 mg/kg/day, a statistically significant increase in the litter incidence of aortic arch supernumerary branch was noted. Though not statistically identified, malpositioned esophagus was noted in 4 foetuses from 3 litters. Incidences of other variations were not dose-dependently changed and/or were known common findings in this strain of rabbit, and therefore considered to be not related to the treatment with the test item.

Based on litter evaluation, a statistically significant increase in the overall incidence of abnormalities considered related to the treatment with the test item was noted at 250 mg/kg/day. At 250 mg/kg body weight occurrence of cleft palate in 3 fetuses (3 litters), of which one case was associated with lip and upper jaw cleft. There were increased incidences of cervical vertebral irregularities (mostly absence, fusions, malformation of vertebral body and/or arches of vertebrae 2 or 3) present in 22 fetuses from 13 litters and abnormalities affecting ribs 1 or 2 and/or costal cartilage (short, interrupted, fused) in 8 foetuses from 6 litters. Low incidences of skeletal abnormalities observed in the control, 1 and 10 mg/kg/day dose groups were considered reflective of normal background and not related to treatment.

Overall, the incidence of skeletal variations across the dose groups was not different from controls. However, statistically significant increased incidences of selected bone and cartilage variations were considered related to the treatment with the test item at 250 mg/kg/day. Structural variations of cervical vertebrae (absent transverse foramen, supernumerary site ventral) affecting 23 foetuses from 11 litters, and cervical vertebral ossification irregularities (incomplete or no ossification, isolated ossification site) affecting 16 foetuses from 10 litters were observed. In addition, at 250 mg/kg/day caudal displacement of the pelvic girdle was noted in 64 foetuses from17 litters. Other skeletal variations increased at 250 mg/kg/day including thoracic rib/costal cartilage variations and slight ossification changes in the skull. There were no statistically significant differences in the incidence of skeletal variations noted at 1 or 10 mg/kg/day compared to the controls.

Based on litter evaluation of bone examinations - ossification stage / supernumerary ribs, an increased incidence of full supernumerary thoracolumbar ribs at 250 mg/kg was observed. An increased incidence of incompletely ossified right and left pubis in 6 and 7 litters, versus none in the control group was also observed.

Based on litter evaluation, the following cartilage variations were considered related to the treatment with the test item; an increase in the incidence of supernumerary costal cartilages at 250 mg/kg/day, increases in the incidence of costal cartilage 1 not reaching the sternum and decreases in the incidence of cartilage 7. At 10 mg/kg/day, a slight but statistical increase in the incidence of supernumerary costal cartilage was considered non-adverse.

Table 7: Intergroup comparison of treatment-related findings in foetuses (litters)

Finding	Dose level of bicyclopyrone (mg/kg/day)			
	0	1	10	250
Number of fetuses (litters) examined	**148 (22)**	**106 (20)**	**114 (20)**	**85 (18)**
External abnormalities				
Jaw and/or palate cleft	0 (0)	0 (0)	0 (0)	3* (3)*
Visceral abnormalities				
Heart: Interventricular septal defect / over-riding aorta /single ventricular chamber (total)	2 (2)	3 (3)	1 (1)	6* (5)
Urogenital(total)	0 (0)	1 (1)	2 (1)	6** (6)**
Kidney and ureter absent	0 (0)	1 (1)	0 (0)	2 (2)
Kidney severely malpositioned caudal (pelvic kidney) and small with or without malrotation	0 (0)	0 (0)	2 (1)	0 (0)
Kidney malpositioned caudal, renal pelvis and ureter severely dilated	0 (0)	0 (0)	0 (0)	1 (1)
Uterine horn absent or threadlike and/or ovary misshapen	0 (0)	1 (1)	0 (0)	4* (4)*
Visceral variations				
Oesophagus malpositioned (right-sided)	1 (1)	1 (1)	1 (1)	4 (3)
Aortic arch supernumerary branch	3 (3)	3 (3)	2 (2)	14** (11)**
Skeletal abnormalities				
Cervical vertebral irregularities (total)	0 (0)	1 (1)	1 (1)	22** (13)**
Vertebra 1 body/arch fused (to exoccipital or adjacent vertebra) / interrupted / misshapen / short	0 (0)	0 (0)	0 (0)	3* (3)
Vertebrae 2, 3 body/odontoid process/arch absent /fused / hemicentric / interrupted / misshapen / small / supernumerary ventral arch or partial ventral arch	0 (0)	0 (0)	1 (1)	21** (12)**
Thoracic rib/costal cartilage 1, 2 fused / interrupted /short	0 (0)	0 (0)	0 (0)	8** (6)**
Skeletal variations				
Cervical vertebra small structural variation (total)	4 (4)	4 (4)	9 (8)	23** (11)**
Vertebral arch 1 transverse foramen absent	4 (4)	4 (4)	9 (8)	20** (11)**
Vertebra 2 supernumerary site ventral	0 (0)	0 (0)	0 (0)	6** (4)**
Cervical vertebra ossification irregularity (total)	0 (0)	0 (0)	3 (3)	16** (10)**
Vertebra 2 odontoid process incompletely ossified or non-ossified	0 (0)	0 (0)	1 (1)	12** (8)**
Vertebral arch 2 isolated ossification site	0 (0)	0 (0)	0 (0)	9** (5)*
Pelvic girdle malpositioned caudal (total)	2 (2)	0 (0)	6 (3)	64** (17)**

Bone examination				
Full supernumerary thoracolumbar ribs right	7 (7)	4 (4)	16* (10)	72** (18)**
Full supernumerary thoracolumbar ribs left	4 (4)	6 (5)	13 (8)	70** (18)**
Incompletely ossified pubis right	0 (0)	1 (1)	2 (1)	9** (6)**
Incompletely ossified pubis left	0 (0)	0 (0)	2 (1)	13** (7)**
Cartilage variations				
Costal cartilage 1 not reaching sternum right	0 (0)	1 (1)	11**(8)**	13** (9)**
Costal cartilage 1 not reaching sternum left	0 (0)	1 (1)	3 (3)	10** (6)**
Costal cartilage 7 not reaching sternum right	39 (13)	27 (14)	26 (12)	3** (2)**
Costal cartilage 7 not reaching sternum left	48 (15)	35 (16)	22* (11)	3** (3)**

*Data were taken from pages 71-81 of the study report
* significant at 5% level, ** significant at 1% level (Fischer's Exact Test)

Table 8: Historical Control Data- Oral Gavage sets (Chosen for similar experimental conditions)

Study Reference	06/05	07/03	07/04
Interventricular septum, Defect perimembraneous regions	1/18 (6%)	3/28 (11%)	1/17 (6%)

Page 204 of MRID 47841998 and page 192 of 47841999

INVESTIGATOR'S CONCLUSIONS: In conclusion, at 250 mg/kg/day, signs of maternal toxicity were noted, including two early deaths, decreased body weight gain and signs of stomach irritation. Fetal developmental effects noted at this dose level consisted of increased post-implantation loss, decreased foetal body weights, and external, visceral and skeletal effects. Fetal effects at 10 mg/kg body weight/day were confined to increases in supernumerary ribs and costal cartilage that were considered non-adverse.

REVIEWER'S COMMENTS: This study was a follow up study to the first developmental Himalayan rabbit study (MRID 47841998) with the intention of increasing the likelihood of establishing a developmental NOAEL as there are a myriad of fetal effects in both studies. The intent was also for the two studies to be considered together.

When looking at the effects in foetuses in the first study, there appeared to be a treatment related effect in the heart described as "Interventricular Septal Variations," which was previously the basis for setting the LOAEL at the lowest dose tested in that study. However, after examination of the historical control data in both the 47841998 and this 47841999 study, there appears to be no dose response for this effect. Furthermore the "total variations" were not reported in the original Tier II summary for this 47841999 study and are depicted in the following table:

Bicyclopyrone-mediated fetal heart effects in Himalayan Rabbits - Interventricular Variations (per litter basis)					
Dose Levels (mg/kg/day)	**0**	**1**	**10**	**50**	**250**
47841998	5/21	NA	8/21	10/19	19/21
47841999	15/22	6/20	10/20	NA	13/18
Total	20/43	6/20	18/41	10/19	32/39
Percent	47%	30%	44%	50%	82%

Data are the sum of sets from the 47841998 (page 65) and 47841999 (page 75)

In a subsequent report submitted by Syngenta (MRID 49383701), changes to the micro-dissection method used for looking at changes to the fetal heart were described. Specifically

Syngenta revised the Stuckhardt and Poppe method which allowed for better viewing of the above discussed Interventricular Septal variations, which were later in part determined to be an artifact of the revised dissection technique.

The report also described how variations such as those observed in the Himalayan rabbit fetal heart are known to be spontaneous and resolve themselves. Furthermore, characterization is provided suggesting that this species is not scientifically the best model for assessing developmental toxicity.

The heart variations at the high-dose in this study do show significant increased incidences of variations in the heart over background levels which are believed to be treatment-related, and the developmental LOAEL will now be set at this dose also for the many observed visceral and skeletal effects at 250 mg/kg/day. The NOAEL will be set at 10 mg/kg/day.

Based upon the results in this study, the maternal LOAEL is 250 mg/kg/day based upon signs of maternal toxicity including two early deaths, signs of stomach irritation, and an increase incidence of post-implantation loss. The maternal NOAEL is 10 mg/kg/day.

Based upon the effects of this study, the developmental LOAEL is 250 mg/kg/day based upon multiple external, visceral and skeletal abnormalities, and multiple visceral variations, skeletal, bone variations and cartilage variations. The developmental NOAEL is 10 mg/kg/day.

APVMA/OCS (Australia) considers the increased resorptions to be fetal as opposed to maternal effects. APVMA agrees with the identified maternal and foetal LOAELs, noting that the skeletal variations identified at the LOAEL were not observed in the first Himalayan rabbit study (see above). APVMA notes that the skeletal variations at the proposed LOAEL were flagged as treatment-related across the developmental studies where a clear treatment-related effect was identified. However, the effects are not considered teratogenic, and APVMA does not consider the chemical to be a developmental toxicant in rabbits.

This study is classified as totally reliable (**acceptable/guideline)** and satisfies the registrants' need (OPPTS 870.3700b; OECD 414) for a developmental toxicity study in rabbits. EPA, PMRA (Canada), AMPVA/OCS (Australia) agree on the classification, but not the regulatory decision for this study.

References:

J.G. Wilson: In: Teratology: Principles and Techniques. Eds., J.G. Wilson and J. Warkany, University of Chicago Press, pp. 265-277 (1965)
Modification of M. Inouye: Differential staining of cartilage and bone in fetal mouse skeleton by Alcian blue and Alizarin red-S. Congenital Anomalies 16, pp. 171-173 (1976)

(Whitlow S, 2012)

Appendix

STUDY TYPE: Dose Range-finding Prenatal Developmental Study (rabbit). Not conducted to any specific regulatory guidelines but based on OECD 414 (2001): OPPTS 870.3700 (1998): JMAFF 12 NohSan No. 8147 (2000)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Whitlow S, 2012. NOA449280: Dose range finding prenatal developmental toxicity study in the Himalayan rabbit. Harlan Laboratories Ltd., (former RCC Ltd.), Wölferstrasse 4, 4414 Füllinsdorf, Switzerland. Laboratory Report No. B50523, 19 September 2012. Unpublished. Syngenta File No. NOA449280_11298 MRID 47841997.

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

Not conducted to any specific regulatory guidelines.

EXECUTIVE SUMMARY

The purpose of this study was to assess the effects of bicyclopyrone (purity 94.5%) on the pregnant female and the embryonic and foetal development when administered orally, by gavage, once daily to mated young adult female Himalayan rabbits from day 5 through to day 27 *post coitum*. The results of this study were used to establish suitable dose levels for a subsequent main prenatal developmental toxicity study in the rabbit.

Each group consisted of 10 mated female rabbits. Bicyclopyrone was administered at dose levels of: 0 (vehicle control), 10, 50, 250 mg/kg body weight/day. A standard dose volume of 4 mL/kg body weight with a daily adjustment to the actual body weight was used. Control animals were dosed with the vehicle alone (0.5% w/v aqueous CMC high viscosity).

All surviving females were killed on day 28 *post coitum* and the foetuses were removed by Caesarean section. Examination of dams and foetuses was performed in accordance with international recommendations.

All female animals survived until scheduled necropsy. No clinical signs or ophthalmoscopic effects were noted in any dams. There was no treatment related effect on body weight or food consumption.

At 250 mg/kg/day, the post-implantation loss was statistically significantly increased with 37.1% of implantation sites (37.1% embryonic resorptions). Thus, the mean number of total foetuses was statistically significantly decreased to 62.9% of implantation sites. At 10 and 50 mg/kg/day, pre-implantation loss, implantation rate, post implantation loss, and the number of living foetuses, respectively, was considered to be not influenced by treatment with the test item.

At necropsy, one dam at 10, 50, and 250 mg/kg/day were noted with crateriform elevations or depressions with increasing diameters at the pylorus area that were interpreted as beginning ulcerations. All other macroscopic findings were considered to be within the range of normal background alterations.

Based upon the results of this study, the maternal LOAEL is 250 mg/kg/day based upon a statistically significant increase in post-implantation loss, and an increase in resorptions. The maternal NOAEL is 50 mg/kg/day.

There were no effects on foetal sex ratios or mean foetal body weight. External examination of foetuses did not reveal any test item-related findings. Treatment-related increases in the overall incidence of visceral abnormalities were observed at 250 and 50 mg/kg/day. At 250 mg/kg/day, the majority of affected foetuses exhibited interventricular septal defect (8 foetuses in 5 litters) and at 50 mg/kg/day, 3 of the 4 foetuses with abnormalities exhibited interventricular septal defects.

Skeletal abnormalities were dose-dependently noted at 50 and 250 mg/kg/day as multiple cervical vertebrae misshapen, supernumerary or fused, cervicothoracic vertebrae fused, thoracic vertebra absent, thoracolumbar vertebrae fused, scoliosis, and rib interrupted or short, and were considered test item-related.

The bone variations were noted with dose-dependency and mostly outside the range of historical reference data at 250 mg/kg/day. These were considered likely test item-related. Skeletal examination (stage of development) of foetuses revealed a mostly dose-dependently increased ossification of sternebra 5, but incompletely or non-ossified sternebra 1, dose dependently increased supernumerary ribs, and decreasing additional ossifications of humerus, femur and tibia bones. These findings were considered likely test item-related.

At 250 mg/kg/day, one foetus was noted with severely dilated lateral ventricles of the brain, bilateral (internal hydrocephaly) that was considered test item-related.

Cartilage abnormalities were dose-dependently noted at 50 mg/kg/day (1 foetus) and at 250 mg/kg/day (11 foetuses in 7 litters) and were considered test item-related. Common cartilage variations were dose-dependent and at 250 mg/kg/day outside the historical control data. Corresponding to the skeletal examination, the incidence was also considered likely to reveal test item related effects.

Based upon the effects in this study, the developmental LOAEL is 10 mg/kg/day based upon an increase in the incidence of skeletal variations. The developmental NOAEL was not observed.

This study is classified **acceptable/non-guideline** and satisfies the intent of the guideline requirement (OPPTS 870.3700b; OECD 414) for a range-finding developmental toxicity study in rabbits.

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar J. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) Date: 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) Date: 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Multigeneration Reproduction Study (rat) OECD Guideline No. 416 (2001): EPA OPPTS 870.3800 (1998): JMAFF, 12 NohSan No. 8147 (2000)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Davies S and Penn L, 2012. NOA449280: Oral (dietary) multigeneration study in the rat. Sequani Ltd., Herefordshire, UK. Laboratory Report No. BFI0004, 13 September 2012. Unpublished. (Syngenta File No.NOA449280/11301). MRID 47842127

SPONSOR: Syngenta Limited, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a 2-generation reproduction study in rats (MRID #47842127), bicyclopyrone (NOA449280, purity 94.5%) was administered to young adult, Crl:WI (Han) rats. Four groups of 25 male and 25 females (P generation) received bicyclopyrone in the diet for 10 weeks at dose levels of 0, 25, 500 or 5000 ppm (0, 2.15/2.65, 43.65/52.7, 435.5/534 mg/kg/day [M/F]) and were then paired (one male with one female) for mating. Formulated diet was available for the males throughout the mating period until necropsy and to females during the mating period, gestation and lactation and up to termination at Day 21 of age of the F1 generation.

The F1 generation (25 males and 25 females per group) was selected from the weaned F1 litters. Formulated diet was available from birth and continued to be available through maturation, pairing (approximately 14 weeks old) and until necropsy for the males and through gestation until Day 21 of age of the F2 generation. The F2 litters received the formulated diet from birth until necropsy on Day 21 of age.

The P and F1 generation parental animals and 3 pups per sex per litter (F1 and F2), where possible, were killed and subjected to necropsy. Any remaining unselected pups were killed and discarded without necropsy.

Clinical signs, ophthalmoscopy, body weights, food consumption, fertility and mating performance, organ weights and macroscopic abnormalities at necropsy were recorded for all parental animals. Microscopic examination of selected organs was conducted. Litter size, clinical condition, ophthalmoscopy, growth and development to weaning, organ weights and macroscopic abnormalities at necropsy were recorded for the offspring.

The parental effects are as follows:

There were no treatment-related mortalities in parental animals.

At 25 ppm in both the P and F1 males, there was an increased incidence of vascular keratitis (60 and 88%) compared to 0% in the controls. For the P males, there was a minor increase in the incidence of corneal opacity and roughness (12%). Regarding macroscopic findings at this dose, there was an increased incidence of opaque/pale colour and red striations in the eyes of the P and F1 males (24 and 52%). In both the P and F1 males, there was an increased incidence of pelvic dilation of the kidney (16 and 32%). For females this increased incidence of pelvic dilation of the kidney was 12%, but only in the F1 generation.

At 500 ppm in parental animals, there was an increased incidence of vascular keratitis (80 and 100% for P and F1 males, and 100% for both P and F1 females) compared to 0% in the controls. For the P males, there was an increased incidence of corneal opacity and roughness (20%). For F1 males there was a minor statistically significant decrease in food consumption from days 64-69 (↓9.5%). Body weight gain was reduced in F1 generation males only. Regarding macroscopic findings at this dose, there was an increased incidence of opaque/pale colour and red striations in the eyes of the P and F1 males (56 and 40%). For the P and F1 females, these values were 8 and 24%. Finally there was an increase incidence of pelvic dilation of the kidney (20 and 64%) in males. For females this increased incidence of pelvic dilation was 40%.

At 5000 ppm in the parental animals, there was an increased incidence of vascular keratitis (84 and 100% for P and F1 males, and 72 and 100% for P and F1 females) compared to 0% in the controls. For the P generation males and females, there was an increased incidence of corneal opacity and roughness (20% and 36%). For the P generation males and females, there was a statistically significant decrease in the absolute body weights pre-pairing (↓6-10% from 36 to 120 days for males and ↓6-7% from 36 to 71 days for females), with associated decreases in body weight gain during pre-pairing. For the P parental females, there were statistically significant decreases in absolute body weights during gestation (↓14%) and lactation (25%). For the F1 parental males and females, there was a statistically significant decrease in the absolute body weights pre-pairing (↓11-14% from 1 to 120 days and ↓6-11% from 1 to 64 days). For the F1 females, there were statistically significant decreases in absolute body weights during gestation (↓6-8%) and lactation (↓6%). Decreases in body weight gains and food consumption all correlated with the decreased absolute body weights. Regarding macroscopic findings at this dose, there was an increased incidence of opaque/pale colour and red striations in the eyes of the P and F1 males (72 and 36%). For the P and F1 females, these values were 8 and 24%. Consistent with the lower dose groups for both the P

and F1 males, there was an increase incidence of pelvic dilation of the kidney (24 and 40%). For females this increased incidence of pelvic dilation was 48% in the F1 generation only.

Based upon these effects, the parental LOAEL is 25 ppm (2.15/2.65 [M/F]) based upon ocular effects (corneal opacity and vascular keratitis in P and F1 males) and an increased incidence of pelvic dilation of the kidney (F0 and F1 males and F1 females). The parental NOAEL was not established.

The offspring effects are as follows:

At the 25 ppm dose, there were no treatment related effects.

At the 500 ppm concentration, there was a statistically significant decrease in mean absolute body weights (↓11%) and body weight gains (↓11%) of males in F1 litters. There was also a decrease in the mean body weight gains of female pups from days 1-21 (↓11%). In both F1 and F2 litters, there was an increased incidence of corneal opacity & roughness (≥36% in both sexes) and vascular keratitis (≥9% in both sexes).

At the 5000 ppm concentration, there was a statistically significant decrease in mean absolute body weights and body weight gains in males of the F1 and F2 litters (↓13% and ↓15%, and ↓12 and ↓13%). There was also a statistically significant decrease in mean absolute body weights and body weight gains in females of the F1 and F2 litters (↓13% and ↓17%, and ↓13% and ↓14%). In both F1 and F2 litters, there was an increased incidence of corneal opacity & roughness (≥36% in both sexes) and vascular keratitis (≥18% in both sexes).

Based upon these effects, the offspring LOAEL is 500 ppm (43.65/52.7 mg/kg/day [M/F]) based upon decreased absolute body weights in the F1 generation, and an increased incidence of litters with ocular effects (corneal opacity and roughness, and vascular keratitis) in the F1 and F2 generations. The offspring NOAEL is 25 ppm (2.15/2.65 mg/kg/day [M/F]).

The reproductive effects are as follows:

At the 25 and 500 ppm doses, there was a treatment related, minor statistically significant increases on the day of preputial separation (↑5%) in the F1 pups. While these observations may be treatment related, in isolation they are not considered adverse due to their small magnitude and absence of any other sexual maturation related findings in offspring at these doses.

At 5000 ppm bicyclopyrone, there were effects on various sperm parameters; velocity of actual path (↓9%), straight line velocity (↓13%), and miscellaneous abnormalities (↑117%). In the F0 generation, there was a statistically significant decrease in mean precoital interval (↓26%). Finally, there was a treatment related, statistically significant treatment related increase on the day of preputial separation (↑9%) in the F1 pups, but was considered to be of low toxicological concern.

Based upon these effects, the reproductive LOAEL is 5000 ppm (435.5/534 mg/kg/day [M/F]) based upon changes in sperm parameters in the F2 generation, and a decrease precoital interval and an increased time for preputial separation in the F1 generation. The reproductive NOAEL is 500 ppm (43.65/52.7, mg/kg/day [M/F]).

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the need for a two-generation reproduction study OPPTS 870.3800 [§83-4]; OECD 416 in the rat.

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, Flagging and Quality Assurance statements were provided.

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown beige powder
Lot/Batch number:	SEZ3AP006/MILLED
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Stable (stored at < 30°C; light protected, dry place)
Structures:	

Vehicle and/or positive control: The test substance was administered SDS CRUK/VRF1 powdered rodent diet (supplied by Charles River (UK) Limited, Margate, Kent, England, UK).

Test Animals:

Species	Rat
Strain	Crl:WI (Han)
Age/weight at dosing	Approximately 6 weeks (P); 25 days (F1) / 169.4-173.7 g (males, group mean range); 150.4-156.9 g (females, group mean range) (P). 58.8-69.5 g (males, group mean range); 55.6-65.5 g (females, group mean range) (F1)
Source	Charles River (UK) Limited, Margate, Kent, England, UK
Housing	Five per cage by sex in stainless steel grid-bottomed cages suspended over paper-lined trays for F0 generation animals and weaned, selected F1 generation animals during acclimatisation (F0 generation) and until pairing for mating, and also for males after mating. During pairing for mating 1 male and 1 female from the same dose group were housed together in polypropylene grid-bottomed cages suspended over paper-lined trays. Towards the end of gestation, females were individually housed in solid-bottomed cages with dust free shavings provided as bedding. The females remained in these cages together with their litters until weaning. Solid-bottomed cages were used to house the F1 and F2 generation litters (1 litter per cage) after weaning and until selection or necropsy.
Acclimatisation period	8 days
Diet	SDS CRUK/VRF1 powdered rodent diet (Charles River (UK) Limited, Margate, Kent, England, UK) *ad libitum*
Water	Mains tap water *ad libitum*
Environmental conditions	Temperature: 18-25°C Humidity: 34-75% Air changes: Not reported Photoperiod: 12 hours dark / 12 hours light

Study Design and Methods:

In-life dates: Start: 16 May 2008 (start of dosing) End: 02 July 2009 (completion of pathology).

Mating procedure: One male was caged with one female from the same test group until sperm cells were observed in vaginal smears taken daily during the mating period for up to 14 days.

After successful mating, each pregnant female was housed individually in grid-bottomed cages over paper-lined trays. Towards the end of gestation, females were individually housed in solid-bottomed cages with dust free shavings provided as bedding. The females remained in these cages together with their litters until weaning.

Study schedule: Four groups of 25 male and 25 female rats (F0 generation) received NOA449280, in the diet for 10 weeks and were then paired (one male with one female) for mating. Formulated diet was available for the males throughout the mating period until necropsy and to females during the mating period, gestation and lactation and up to termination at Day 21 of age of the F1 generation. The animals were provided with diet containing the test article at dose levels of 0, 25, 500 or 5000 ppm.

The F1 generation of 25 males and 25 females per group (at least 1 male and 1 female per litter, where possible) was selected from the weaned F1 litters. The selection of the F1 animals was done on a body weight basis. Formulated diet was available from birth and continued to be available through maturation, pairing (approximately 14 weeks old) and until

necropsy for the males and through gestation until Day 21 of age of the F2 generation. The F2 litters received the formulated diet from birth until necropsy on Day 21 of age.

The F0 and F1 generation parental animals and 3 pups per sex per litter (F1 and F2), where possible, were killed and subjected to necropsy. Any remaining unselected pups were killed and discarded without necropsy.

Animal assignment: Parental (P) generation animals were allocated to dose groups separately by sex using a body weight stratification technique. For the F1 generation animals, 25 male and 25 female pups per group were randomly selected from the weaned F1 litters (one of each sex from each of 25 weaned litters, where possible). See table 1.

Table 1: Animal numbers and treatment groups

	Dietary Concentration of bicyclopyrone (ppm)							
	Males				Females			
generation	0	25	500	5000	0	25	500	5000
P	1-25	26-50	51-75	76-100	101-125	126-150	151-175	176-200
F1	201-225	226-250	251-275	276-300	301-325	326-350	351-375	376-400

Animal assignments were taken from page 28 of the study report

Dose selection rationale: The dose levels were selected after examining existing toxicity data and on the basis of results from a preliminary study (BFI0003) performed at Sequani Limited. In this study, dietary administration of bicyclopyrone to the Crl:WI (Han) strain of rat, at dose levels of 25, 500, 2500 and 5000 ppm for 10 weeks before pairing, during pairing and through to weaning of the F1 generation, was generally well tolerated (see appendix). At 2500 and 5000 ppm an effect on food consumption during lactation was apparent as were F0 and F1 body weight effects and smaller litter sizes. A dose level of 25 ppm was considered by the study director to be the No Observed Adverse Effect Level (NOAEL) for maternal treatment and for pup development to weaning.

Diet/dosage preparation and analysis: A stock premix was prepared at the concentration of the highest dose group (5000 ppm), by incorporating a specified amount of test article into the appropriate quantity of plain SDS CRUK/VRF1 diet which was mixed in a double cone blender. For each of the lower dosage groups separately, a specified amount of premix stock was diluted with a further quantity of plain diet and mixed in a double cone blender. Diet preparations were prepared as required at appropriate intervals according to stability.

The homogeneity and stability of the dietary preparations for up to 5 weeks when stored at room temperature and for 10 weeks when stored frozen, at approximately -18°C were assessed. Achieved concentration and homogeneity were assessed in weeks 1, 8, 16, 24 and 32 of the study.

Results: The results for dietary concentrations of 25, 500 and 5000 ppm analysed during weeks 1, 8, 16, 24 and 32 of the study showed the diets to be accurate and homogenous. No test article was found in the control group samples. A summary of the validation numbers is as follows:

Concentration analysis results: The test item concentration in all samples was 102 % to 105 % of the nominal concentration.

Homogeneity results: Homogenous distribution was demonstrated because single results found did not deviate more than 4.1 % (<15%) from the corresponding mean.

Stability results: The dosing formulations were considered to be stable for at least 32 weeks under storage conditions.

The analytical data indicate that the test substance was homogeneous and stable in the suspensions and that the variation between the nominal and actual dosages to the animals was acceptable.

Observations:
Parental animals:
Mortality and clinical observations: All animals were examined twice daily for mortality and morbidity. The uteri of females that failed to deliver a litter were stained with ammonium sulphide to confirm pregnancy status. All visible signs of reaction to treatment were recorded daily. In addition, each animal was given a detailed examination once a week, after the commencement of exposure to the test article.

Body weight: Body weights of the males were recorded at weekly intervals throughout the study until necropsy. Body weights of the females were recorded at weekly intervals during the prepairing dosing period, twice weekly from the start of the pairing period (but not reported) and on Days 0, 4, 7, 10, 14, 17 and 20 of gestation and Days 1, 4, 7, 10, 14, 17 and 21 of lactation. Cumulative bodyweight gains were reported for each period of the study.

Food consumption: The amount of food consumed by the animals in each cage was recorded at weekly intervals for males and females during the pre-pairing period. Food consumption of the females was also recorded for Days 0-4, 4-7, 7-10, 10-14, 14-17 and 17-20 of gestation and for Days 1-4, 4-7, 7-10, 10-14, 14-17 and 17-21 of lactation.

Ophthalmoscopy: All P parental males and females were examined during Week 10 prior to pairing for mating. The examinations were performed using direct and indirect ophthalmoscopy after previous use of a mydriatic agent.

Oestrous cycle monitoring: For 21 days before the start of the pairing period, vaginal smears were taken daily by lavage. Each smear was examined under light microscopy and the stage of the oestrous cycle was recorded. The stage of the oestrous cycle was determined by the type of cell present. In addition, a vaginal smear was taken from each female on the day of scheduled termination and examined.

Parturition observations: Where possible, the onset and completion of parturition was recorded. Females were observed at least 3 times daily (the beginning, middle and end of the working day), commencing when the first animal was on Day 21 of gestation and finishing when the last mated female was on Day 25 of gestation. The duration of gestation was calculated from Day 0 of gestation until the onset of parturition.

The females were allowed to rear their offspring to weaning on Day 21 of age. The nursing and nesting behaviour of the maternal animals was observed and recorded.

Sperm parameters: Immediately after weighing, the right cauda epididymis from all males was used for sperm examination. Data recorded included straight line velocity (VSL), actual

path velocity (VAP) and percentage motile sperm. Samples of sperm were also evaluated for sperm total count and morphology. For morphology a smear was prepared and the sperm were examined by light microscopy including examination for headless, tailless, reduced hook and miscellaneous abnormalities (to include gross abnormalities such as two-headed sperm). Homogenisation-resistant testicular spermatid count was conducted on the right testis. The left testis was fixed, for approximately 24 hours, in Bouin's fixative and then transferred to neutral buffered formaldehyde.

Litter observations: The litters were not standardized. The following parameters were examined:

Observation	Frequency
Litter size and sex:	F1 and F2 offspring: Total litter size was recorded after completion of littering. Litter size was recorded daily thereafter and pups were sexed on days 1, 4, 7, 14, 17 and 21 of age.
Clinical observations and mortality	F1 and F2 offspring : All pups were examined after the completion of littering for malformations and the pups were examined daily.
Pup body weights	F1 and F2 offspring : Pups were weighed individually on Days 1, 4, 7, 14, 17 and 21 of age
Anogenital distance	F2 offspring: Recorded on day 1 of age
Ophthalmoscopy	F1 offspring: 1 pup/sex/ litter, where possible, were randomly selected and examined on day 19 ± 3 of age; F1 parental animals examined again during week 9 and week 10 prior to pairing for mating. The examinations were performed using direct and indirect ophthalmoscopy after previous use of a mydriatic agent.
Sexual development	F1: All selected F1 males were examined daily for balanopreputial separation from the time the first male reached Day 35 of age until all males showed balanopreputial separation. Balanopreputial separation was assessed by gently stretching the skin of the penis and observing whether the glans had separated from the prepuce. On the day of attainment the body weight of the animal was recorded. All F1 females selected for mating were examined daily for vaginal perforation from the time the first female reached Day 28 of age until all the females showed vaginal perforation. On the day of attainment the body weight of the animal was recorded.
Pup necropsy	F1 and F2 offspring: A necropsy was conducted on all pups prematurely killed or found dead. The pups were killed by an intraperitoneal injection of sodium pentobarbitone solution (for those up to the age of 14 days) or exposure to carbon dioxide gas in a rising concentration (for older pups). The thoracic and abdominal cavities were opened by a ventral mid-line incision and the major organs examined. Organs or tissues showing macroscopic lesions were removed and fixed in neutral buffered formaldehyde.

Investigations *post mortem:*
Parental animals: All surviving parental males were killed once successful littering had been completed and the need for a second mating had been considered. At termination, the males were weighed and a necropsy was performed.

Gross necropsy consisted of opening the abdominal cavity and exsanguinating the animal from the caudal vena cava. Macroscopic examination was performed by observing the appearance of tissues in situ from the cranial, thoracic and abdominal cavities. The uteri of any apparently non-pregnant females were stained with ammonium sulphide to confirm pregnancy status. No histopathology was performed on stained uteri. For pregnant females the uterus was examined and the number of implantation scars in each uterine horn recorded.

The following organs were weighed: Adrenal glands, brain, left epididymis (including cauda), right epididymis (including cauda), kidneys, liver, ovaries, pituitary gland, prostate gland, seminal vesicles (incl. coagulating gland with their fluids), spleen, left testis, right testis, thyroids (incl. parathyroids), and uterus (incl. uterine cervix and oviducts). Paired organs were weighed together, except testes and epididymides which were weighed separately

Microscopic examination: The following tissues weighed and examined microscopically: Adrenal glands, left epididymis (including cauda), kidneys, liver, ovaries, pituitary gland, prostate gland, seminal vesicles (incl. coagulating gland with their fluids), site of mammary gland (females only), left testis, thyroids (incl. parathyroids), uterus (incl. uterine cervix and oviducts), vagina and all gross lesions All processed tissues were examined by light microscopy. The number of ovarian follicles in F1 females was quantified.

Offspring: The F1 offspring not selected as parental animals and all F2 offspring were sacrificed at 21 days of age. Pups were killed by exposure to carbon dioxide gas in a rising concentration. Three male and 3 female pups per litter were examined and the remaining unselected F1 and F2 pups were killed and discarded without necropsy.

Thoracic and abdominal cavities opened by a ventral mid-line incision and the major organs examined. Organs or tissues showing macroscopic lesions were removed and fixed in neutral buffered formaldehyde.

A terminal body weight was recorded and the weights of the brain, liver, kidneys, spleen and thymus were recorded for 1 male and 1 female pup per litter, where possible (selected from the 3 per sex per litter for macroscopic examination); paired organs were weighed together. Weighed organs, together with any grossly abnormal tissue, were retained in 10% neutral buffered formaldehyde.

Statistics: Data were processed to give group mean values and standard deviations where appropriate. Data from each sex and data for each generation were analysed separately. Where the data allowed, the following methods were used for statistical analyses: Analysis of Variance, Analysis of Covariance, Analysis of Variance following Double Arcsine Transformation, Dunnett's Test and Fisher's Exact Test.

Indices:
Reproductive indices: The following indices were calculated, copulation index, fertility index, gestation index and post implantation loss.

Offspring viability indices: The following indices were calculated from breeding and parturition records and lactation records: Live birth index, viability index and cumulative survival index.

RESULTS

Parental animals:
Mortality and clinical signs:
P: There were no treatment-related deaths. Treatment-related ocular findings of corneal opacity and roughness and vascular keratitis were seen at all dose levels in males and at 500 and 5000 ppm in F0 parental females. There was one P male on Day 88 due to adverse clinical condition caused by mammary carcinoma and that it was unrelated to treatment. See table 2.

F1: Treatment related vascular keratitis was seen at all dose levels in males and at ≥ 500 ppm in males and females.. See table 2.

Table 2: Incidence by animal of opacity and roughness and vascular keratitis - Parents

	Dietary concentration of bicyclopyrone (ppm)			
	0	**25**	**500**	**5000**
P parent animals (25/sex/group)				
Corneal opacity & roughness - males	0	3	5	5
Corneal opacity & roughness - females	0	0	1	9
Vascular keratitis – males	0	15 (60%)	20 (80%)	21 (84%)
Vascular keratitis - females	0	0	25 (100%)	18 (72%)
F1 parent animals (25/sex/group)				
Corneal opacity & roughness - males	0	2	0	0
Corneal opacity & roughness - females	0	4	0	0
Vascular keratitis – males	0	22 (88%)	25 (100%)	25 (100%)
Vascular keratitis - females	0	0	25 (100%)	25 (100%)

Data were taken from page 46 of the study report

Bodyweight:

F0 generation: A statistically significantly decreased body weight was noted in males at 5000 ppm from day 36 (↓6-10%). Body weight gains were statistically significantly lower than controls throughout the treatment period (↓14-21%). See table 3 and 5.

Body weights of females at 5000 ppm were statistically significantly lower than controls from day 36 and remained lower throughout the rest of pre-pairing and gestation (↓6-8%). Body weight gains for 5000 ppm females were statistically significantly lower during most of the pre-pairing and gestation periods, when compared with controls (↓14-23%). During lactation, 5000 ppm females had lower body weights (↓7-10%), compared with controls, with statistically significant decreases in body weight gain in 5000 ppm females over days 0-14 of lactation (↓25%). See tables 3, 4 and 5.

There were no treatment-related effects on body weight or body weight gains in males or females at 25 or 500 ppm.

F1 generation: Mean body weights and body weight gain for males given 5000 ppm bicyclopyrone, were statistically significantly lower throughout the F1 generation treatment period compared with controls (↓10-14% and ↓11-15%, respectively). Males given 500 ppm bicyclopyrone had statistically significantly lower body weights and body weight gains from day 29 onwards (↓6-8% and ↓7-8%, respectively). There was no treatment-related effect at 25 ppm in males. See table 6.

Mean body weights were statistically significantly lower for 5000 ppm females throughout the pre-pairing period and for the first 14 days of gestation (both ↓6-11%). Body weights were lower than controls, occasionally achieving statistical significance, throughout the rest of the gestation and lactation periods (↓6%). Females given 5000 ppm continued to gain body weight towards the end of lactation whereas controls lost weight. Body weight gains for 5000 ppm females were similar to controls throughout the pre-pairing period, gestation and lactation with the exception of days 1 to 8 of the pre-pairing period (↓8%) and days 0-14 of lactation (↓0-14%). Hence, growth rates in females were similar to controls and the difference in body weight reflected the lower body weight at the beginning of the pre-weaning period. There were no treatment-related effects in females at 25 or 500 ppm. See tables 6, 7 and 8.

Table 3: Intergroup comparison of premating absolute bodyweights – P parents

Study days	Dietary Concentration of bicyclopyrone (ppm)							
	Males				Females			
	0	25	500	5000	0	25	500	5000
P (pre-pairing)								
1	173.1 ± 13.1	169.4 ± 13.0	173.7 ±13.1	173.4 ± 14.9	151.8 ± 10.9	150.4 ± 12.1	156.9 ± 12.5	152.5 ± 12.3
8	217.7 ± 15.8	212.6 ± 16.7	216.2 ± 17.5	208.5 ± 16.0	170.0 ± 11.3	171.4 ± 15.8	175.0 ± 13.4	166.4 ± 12.9
15	260.9 ± 18.0	256.0 ± 20.5	261.0 ± 20.7	249.1 ± 18.0	187.5 ± 10.7	187.7 ± 19.0	191.4 ± 15.9	177.9 ± 13.0
22	287.6 ± 24.3	283.4 ± 25.5	292.2 ± 23.2	274.1 ± 20.4	195.5 ± 10.9	196.9 ± 19.4	199.5 ± 17.1	185.7 ± 13.9
29	310.1 ± 28.0	304.8 ± 30.7	314.7 ± 25.5	292.8 ± 23.6	203.7 ± 11.7	204.1 ± 20.2	209.5 ± 16.7	194.6 ± 14.9
36	328.4 ± 33.0	321.8 ± 33.5	331.3 ± 27.3	307.3* ± 26.7 (↓6%)	213.9 ± 13.8	214.4 ± 21.3	217.8 ± 15.8	200.9* ± 17.5 (↓6%)
43	345.4 ± 34.9	335.8 ± 36.7	346.7 ± 29.7	320.1* ± 28.3 (↓7%)	220.6 ± 12.4	222.7 ± 23.1	224.3 ± 17.0	206.8* ± 17.0 (↓6%)
50	359.3 ± 37.9	345.7 ± 39.4	359.2 ± 31.6	328.0** ± 30.4 (↓9%)	223.4 ± 13.3	226.2 ± 23.7	226.1 ± 16.8	209.7* ± 16.4 (↓6%)
57	371.6 ± 38.5	356.5 ± 42.5	368.7 ± 31.9	338.0** ± 31.8 (↓9%)	228.7 ± 11.5	230.1 ± 23.3	231.6 ± 16.7	214.2* ± 17.8 (↓6%)
64	381.9 ± 39.2	364.6 ± 44.2	378.1 ± 34.7	345.4** ± 33.7 (↓10%)	234.0 ± 12.6	236.2 ± 24.2	236.1 ± 16.7	218.3** ± 18.5 (↓7%)
71	391.1 ± 39.1	371.8 ± 46.5	386.5 ± 35.9	354.1** ± 35.7 (↓9%)	235.2 ± 12.5	237.6 ± 25.4	238.5 ± 18.7	219.5* ± 17.9 (↓7%)
78	389.8 ± 40.6	371.5 ± 46.1	385.5 ± 36.1	354.8** ± 33.8 (↓9%)	NA	NA	NA	NA
85	402.0 ± 41.1	381.2 ± 47.2	396.4 ± 37.4	363.3** ± 35.9 (↓10%)	NA	NA	NA	NA
92	407.8 ± 40.6	386.9 ± 48.5	400.3 ± 34.2	370.8** ± 38.5 (↓9%)	NA	NA	NA	NA
99	417.8 ± 42.2	397.0 ± 49.5	406.7 ± 33.9	377.2** ± 39.8 (↓10%)	NA	NA	NA	NA
106	424.1 ± 43.7	400.4 ± 51.0	413.4 ± 33.8	382.1** ± 38.3 (↓10%)	NA	NA	NA	NA
113	430.0 ± 43.2	406.7 ± 51.7	418.6 ± 33.7	387.4** ± 40.0 (↓10%)	NA	NA	NA	NA
120	434.5 ± 43.4	411.6 ± 53.1	423.6 ± 34.6	389.7** ± 40.7 (↓10%)	NA	NA	NA	NA

Data were taken from pages 76-78 of the study report
*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01
***statistically significantly different from control, p=<0.001

Table 4: Intergroup comparison of lactational and gestational absolute bodyweights – P females

Study days	Dietary Concentration of bicyclopyrone (ppm)							
	Gestation				Lactation			
	0	25	500	5000	0	25	500	5000
0	233.3 ± 13.3	234.8 ± 25.2	238.3 ±17.0	219.3 ± 17.5	261.9 ± 16.7	263.7 ± 28.5	262.6 ± 19.9	241.0** ± 19.9 (↓8%)
4	248.6 ± 14.7	249.4 ± 27.7	250.5 ± 19.3	231.9* ± 20.4 (↓7%)	273.4 ± 18.0	274.6 ± 26.4	270.0 ± 19.0	248.7*** ± 21.2 (↓9%)
7	254.9 ± 14.4	256.6 ± 28.3	257.5 ± 19.8	237.5* ± 20.9 (↓7%)	282.6 ± 19.2	283.0 ± 25.6	279.4 ± 18.8	258.6*** ± 21.3 (↓8%)
10	265.2 ± 15.5	267.2 ± 29.5	265.0 ± 21.1	244.6** ± 21.7	289.1 ± 24.6	285.0 ± 31.4	287.0 ± 18.8	268.8*** ± 23.4 (↓7%)
14	277.4 ± 17.3	276.8 ± 31.8	276.9 ± 21.5	254.8** ± 21.3 (↓8%)	300.8 ± 20.8	293.3 ± 29.5	297.4 ± 22.9	269.6*** ± 21.7 (↓10%)
17	299.8 ± 18.8	302.9 ± 33.1	301.0 ± 24.0	277.1** ± 25.1 (↓8%)	302.3 ± 20.7	298.1 ± 29.3	296.7 ± 24.7	271.2*** ± 22.2 (↓10%)
20/21	336.5 ± 23.7	339.6 ± 38.3	335.4 ± 28.6	308.4** ± 29.4 (↓8%)	295.1 ± 20.7	293.9 ± 29.0	296.7 ± 23.2	269.0*** ± 24.4 (↓9%)

Data were taken from pages 79 and 80 of the study report
*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01
***statistically significantly different from control, p=<0.001

Table 5: Intergroup comparison of bodyweight gain (g) – P Parents

Study days	Dietary Concentration of bicyclopyrone (ppm)							
	Males				Females			
	0	25	500	5000	0	25	500	5000
P (pre-pairing)								
1-8	44.6 ± 4.9	43.1± 6.0	42.5 ± 6.5	35.1*** ± 4.0 (↓21%)	18.2 ± 4.9	21.1 ± 6.3	18.1 ± 4.1	14.0**± 4.2 (↓23%)
1-36	155.3 ± 25.4	152.4 ± 27.0	157.6 ± 20.3	133.9** ± 19 .3 (↓14%)	62.1 ± 12.2	64.0± 13.4	60.8 ± 10.2	48.4*** ± 13.3 (↓22%)
1-64	208.8± 32.1	195.2 ± 37.2	204.4 ± 28.6	172.0*** ± 26.1 (↓18%)	82.2 ± 12.1	85.8 ± 16.6	79.2 ± 11.4	65.8*** ± 13.7 (↓20%)
1-92	234.7± 34.6	217.5± 41.5	227.2± 28.7	197.4*** ± 31.1 (↓16%)	NA	NA	NA	NA
1-120	261.4± 37.6	242.2± 46.7	250.5± 28.4	216.3*** ± 33.1 (↓17%)	NA	NA	NA	NA
P females								
	Gestation				Lactation			
0-7	21.5 ± 5.0	19.9 ± 4.9	19.3± 7.1	18.2 ± 5.8	20.7 ± 7.5	19.4 ± 9.4	15.9 ± 10.9	18.1 ± 10.4
0-14	44.1± 9.4	39.5 ± 6.2	38.6± 8.9	35.5** ± 7.4 (↓20%)	38.9 ± 11.1	29.6*± 12.2 (↓24%)	33.8± 15.7	29.1*± 10.3 (↓25%)
0-20	103.2± 14.9	101.9 ± 15.4	97.1 ± 17.2	89.1* ± 18.2 (↓14%)	33.2± 11.9	30.2 ± 14.7	33.1 ± 12.4	28.5 ± 13.6

Data were taken from pages 81-85, and 152-156 of the study report
*statistically significantly different from control, p=<0.05

**statistically significantly different from control, p=<0.01
***statistically significantly different from control, p=<0.001

Table 6: Intergroup comparison of absolute bodyweights – F1 parents

Study days	Dietary Concentration of bicyclopyrone (ppm)							
	Males				Females			
	0	25	500	5000	0	25	500	5000
1	66.2 ± 7.2	69.5 ± 9.2	63.3 ± 9.4	58.8* ± 10.0 (↓11%)	62.8 ± 7.1	65.5 ± 7.2	59.8 ± 8.3	55.6** ± 9.1 (↓11%)
8	109.0 ± 10.2	114.3 ± 12.4	104.6 ± 11.3	96.8** ± 12.9 (↓11%)	97.4 ± 9.0	101.8 ± 9.4	93.8 ± 9.8	87.4** ± 10.8 (↓10%)
15	153.2 ± 12.8	159.9 ± 14.7	147.2 ± 12.1	137.4*** ± 14.6 (↓10%)	127.4 ± 10.6	131.8 ± 11.0	123.8 ± 10.9	117.0*** ± 9.9 (↓8%)
22	193.8 ± 15.7	198.2 ± 16.6	184.3 ± 14.4	174.2*** ± 17.6 (↓10%)	146.5 ± 11.5	151.4 ± 12.5	143.1 ± 11.0	136.7** ± 9.7 (↓7%)
29	234.8 ± 17.6	237.9 ± 19.6	219.5** ± 16.0 (↓7%)	207.2*** ± 18.2 (↓12%)	166.6 ± 12.9	171.0 ± 16.1	160.8 ± 13.9	153.4** ± 10.8 (↓8%)
36	266.3 ± 19.7	268.8 ± 22.9	248.8** ± 19.0 (↓7%)	236.8** ± 20.1 (↓11%)	181.2 ± 15.0	184.1 ± 17.2	174.6 ± 12.8	168.6** ± 11.3 (↓7%)
43	289.8 ± 23.3	295.5 ± 25.6	271.8* ± 21.3 (↓6%)	259.0*** ± 21.6 (↓11%)	191.4 ± 16.2	195.9 ± 18.8	185.2 ± 11.8	177.9** ± 11.6 (↓7%)
50	307.7 ± 26.5	312.6 ± 28.7	289.3* ± 23.2 (↓6%)	273.8*** ± 23.6 (↓11%)	199.5 ± 16.7	204.6 ± 19.9	195.4 ± 12.0	186.4** ± 13.0 (↓7%)
57	326.8 ± 28.5	331.0 ± 30.7	306.6* ± 25.1 (↓6%)	289.0*** ± 24.1 (↓12%)	208.3 ± 16.5	213.8 ± 21.6	203.8 ± 11.9	196.6* ± 14.0 (↓6%)
64	341.6 ± 29.9	344.8 ± 33.9	316.6** ± 26.8 (↓6%)	298.0*** ± 24.5 (↓13%)	218.8 ± 19.1	222.7 ± 22.2	210.5 ± 11.5	203.2** ± 15.4 (↓7%)
71	348.6 ± 30.2	348.4 ± 32.4	321.4** ± 25.9 (↓7%)	299.9*** ± 25.4 (↓14%)	NA	NA	NA	NA
78	356.6 ± 32.1	359.5 ± 35.6	333.5* ± 30.2 (↓6%)	313.1*** ± 27.4 (↓12%)	NA	NA	NA	NA
85	368.4 ± 33.2	370.2 ± 37.3	344.2* ± 30.6 (↓7%)	322.6* ± 28.8 (↓12%)	NA	NA	NA	NA
92	377.5 ± 34.6	377.4 ± 40.5	351.1* ± 30.4 (↓7%)	329.9* ± 30.3 (↓13%)	NA	NA	NA	NA
99	383.8 ± 37.3	385.4 ± 40.4	357.5* ± 31.0 (↓7%)	334.7*** ± 31.8 (↓13%)	NA	NA	NA	NA
106	393.9 ± 36.9	394.6 ± 41.0	366.6* ± 31.9 (↓7%)	343.1*** ± 32.4 (↓14%)	NA	NA	NA	NA
113	399.3 ± 36.6	398.6 ± 42.1	369.1* ± 31.6 (↓8%)	343.3*** ± 32.3 (↓14%)	NA	NA	NA	NA
120	407.6 ± 37.4	404.1 ± 43.3	375.4** ± 32.5 (↓8%)	349.8*** ± 34.0 (↓14%)	NA	NA	NA	NA

Data were taken from pages 147-149 of the study report
*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01
***statistically significantly different from control, p=<0.001

Table 7: Intergroup comparison of lactational and gestational absolute bodyweights – F1 females

Study days	Dietary Concentration of bicyclopyrone (ppm)							
	Gestation				Lactation			
	0	25	500	5000	0	25	500	5000
F1 (pre-pairing)								
0	219.7 ± 19.0	224.7 ± 23.4	211.8 ± 11.1	201.2** ± 15.6 (↓8%)	245.5 ± 22.9	253.8 ± 26.6	247.6 ± 15.4	236.9 ± 16.5
4	230.5 ± 19.1	235.5 ± 23.1	223.6 ± 11.1	214.1** ± 16.3 (↓7%)	257.6 ± 22.0	261.7 ± 27.1	253.4 ± 15.3	244.9 ± 19.2
7	237.4 ± 22.1	241.5 ± 23.8	230.8 ± 12.2	220.6* ± 15.6 (↓7%)	267.4 ± 25.9	272.1 ± 26.6	264.1 ± 18.2	253.4 ± 18.9
10	247.1 ± 21.3	253.7 ± 24.9	241.9 ± 14.2	230.2* ± 17.6 (↓7%)	277.4 ± 22.7	282.9 ± 27.4	273.3 ± 15.7	260.6* ± 21.2 (↓6%)
14	260.9 ± 22.4	266.8 ± 24.6	257.5 ± 15.2	245.2* ± 19.0 (↓6%)	282.1 ± 20.1	288.0 ± 25.4	279.3 ± 17.1	264.8* ± 21.9 (↓6%)
17	280.7 ± 25.0	288.8 ± 29.3	280.6 ± 17.0	263.5 ± 22.9	280.0 ± 22.6	290.6 ± 23.2	284.7 ± 15.2	266.6 ± 22.9
21	312.7 ± 28.5	325.0 ± 33.3	314.3 ± 19.2	297.8 ± 30.6	275.4 ± 17.2	278.4 ± 25.6	278.9 ± 18.2	267.6 ± 21.9

Data were taken from page 150-151 of the study report
*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01
***statistically significantly different from control, p=<0.001

Table 8: Intergroup comparison of bodyweight gain (g) – F1 Parents

Study days	Dietary Concentration of bicyclopyrone (ppm)							
	Males				Females			
	0	25	500	5000	0	25	500	5000
F1 (pre-pairing)								
1-8	42.8 ± 3.8	44.8 ± 4.2	41.3 ± 3.5	38.0*** ± 4.4 (↓11%)	34.6 ± 3.5	36.3 ± 3.8	34.0 ± 2.8	31.8** ± 2.9 (↓8%)
1-36	200.1 ± 16.2	199.3 ± 16.6	185.5** ± 16.0 (↓7%)	178.1***± 17.4 (↓11%)	118.4 ± 12.5	118.6 ± 15.0	114.8 ± 8.9	113.0 ± 9.4
1-64	275.4 ± 26.5	275.3 ± 28.1	253.2** ± 24.6 (↓8%)	239.2***± 22.7 (↓13%)	156.0 ± 16.8	157.2 ± 20.4	150.6 ± 8.6	147.6 ± 14.1
1-92	311.3 ± 31.3	308.0 ± 35.6	287.8* ± 27.9 (↓8%)	271.1***± 28.2 (↓13%)	NA	NA	NA	NA
1-120	341.3 ± 34.0	334.6 ± 38.6	312.1** ± 30.0 (↓9%)	291.0***± 31.8 (↓15%)	NA	NA	NA	NA
F1 Females								
	Gestation				Lactation			
0-7	17.7 ± 5.3	16.8± 3.9	19.0 ± 4.3	19.4 ± 5.6	21.8 ± 9.3	17.5 ± 8.8	16.4 ± 10.9	16.5 ± 6.5
0-14	41.2 ± 6.4	42.1 ± 5.3	45.7 ± 8.8	44.0 ± 7.7	36.6 ± 11.8	33.4 ± 10.2	32.2 ± 9.7	27.5* ± 11.8 (↓25%)
0-20	93.0 ± 15.6	100.2 ± 13.6	102.4 ± 15.4	96.6 ± 19.2	29.8 ± 10.8	23.7 ± 16.0	31.8 ± 13.2	30.4 ± 10.8

*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01
***statistically significantly different from control, p=<0.001
Data were taken from pages 81-85, and 152-156 of the study report

Food consumption: For all food consumption data, see table 9.

F0 generation: At 5000 ppm, food consumption was slightly lower during the pre-mating period, compared with controls, for males and females, achieving statistical significance over days 1 to 8 for males, and for most weekly intervals from Day 22 for males and females.

During gestation, food consumption was slightly lower for 5000 ppm females, achieving statistical significance over Days 7 to 14 and 14 to 20 of gestation (↓9%).

Though not statistically significant, food consumption during lactation was approximately 10% lower than controls at 500 and 5000 ppm.

F1 generation: At 5000 ppm, food consumption was statistically significantly lower for males, during the pre-mating period, with the exception of days 36 to 43 (↓10-12%). From day 22 of the pre-mating period males at 500 ppm had statistically significantly lower food consumption, with the exception of days 36 to 43.

Food consumption was slightly lower for females given 5000 ppm during pre-pairing (statistically significant at days 1-8 and 22-29).

There was no apparent effect on food consumption for females given bicyclopyrone during gestation.

Females given 500 or 5000 ppm bicyclopyrone had lower food consumption during lactation. This decrease was statistically significant for 5000 ppm females (↓12-16%) throughout lactation and for 500 ppm females over Days 1 to 7 of lactation (↓13%).

Table 9: Intergroup comparison of food consumption (g/cage) – Parents

Study days	Dietary Concentration of bicyclopyrone (ppm)							
	Males				Females			
	0	25	500	5000	0	25	500	5000
P (pre-pairing)								
1-8	816.0 ± 11.8	802.8 ± 35.5	811.4 ± 8.1	738.4*** ± 10.8 (↓10%)	602.4 ± 35.6	602.6 ± 30.6	628.6 ± 25.9	560.2 ± 16.2
36-43	796.2 ± 43.8	752.0 ± 53.8	793.6 ± 37.0	714.2* ± 4.0 (↓10%)	599.4 ± 21.4	609.2 ± 26.3	623.0 ± 33.4	557.8* ± 17.0 (↓7%)
64-71	757.8 ± 23.2	702.0* ± 39.0 (↓7%)	740.6 ± 28.1	689.2** ± 34.4 (↓9%)	557.6 ± 19.7	567.2 ± 34.5	569.2± 16.2	529.8 ± 13.8
P females (gestation)								
	Gestation				**Lactation**			
1-7	137 ± 12	137 ± 15	141 ± 15	131 ± 17	231 ± 32	218 ± 38	212 ± 39	215 ± 38
7-14	156 ± 11	156 ± 18	155 ± 17	142** ± 17 (↓9%)	365 ± 60	351 ± 49	340 ± 66	337 ± 49
14-20	146 ± 15	145 ± 20	144 ± 11	133*± 17 (↓9%)	450 ± 92	427± 66	405 ± 79	407 ± 75
Study days	**Dietary Concentration of bicyclopyrone (ppm)**							
	Males				**Females**			
	0	**25**	**500**	**5000**	**0**	**25**	**500**	**5000**
F1 (pre-pairing)								
1-8	472.0 ± 31.2	494.6 ± 15.2	469.6 ± 27.5	425.0* ± 33.5 (↓10%)	421.6 ± 19.9	439.4 ± 11.0	415.8 ± 24.9	380.0* ± 31.9 (↓10%)
36-43	758.6 ± 119.1	781.8 ± 25.2	723.0 ± 23.6	722.4 ± 17.5	567.8 ± 29.7	554.6 ± 25.4	527.4 ± 32.0	529.6 ± 24.8
64-69	527.0 ± 30.6	503.8 ± 29.1	482.6* ± 11.5 (↓8%)	461.2** ± 17.7 (↓12%)	406.8 ± 9.8	398.8 ± 15.2	378.0 ± 7.2	383.0 ± 31.9
F1 females (gestation)								
	Gestation				**Lactation**			
0-7	136 ± 14	135 ± 10	132 ± 14	128 ± 14	226 ± 35	212 ± 31	197* ± 34 (↓13%)	196** ± 33 (↓13%)
7-14	152 ± 16	153 ± 23	148 ± 17	148 ± 22	384 ± 64	375 ± 43	350 ± 45	324** ± 61 (↓16%)
14-20	137 ± 13	140 ± 15	144 ± 15	140 ± 14	451 ± 74	467 ± 76	414 ± 54	396* ± 81 (↓12%)

Data were taken from pages 157-160 of the study report
*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01
***statistically significantly different from control, p=<0.001

Food conversion:
F0 generation: Group mean food conversion was less efficient during the pre-pairing period for males and females at 5000 ppm, achieving statistical significance over Days 1-36 for females, Days 36-71 for males and Days 1-71 for both sexes. Males given 25 ppm had slightly lower food conversion efficiency values over Days 36-71 of pre-pairing, however, values were similar to controls over the entire pre-pairing period and thus this isolated difference is considered not to be related to treatment. There were no treatment-related effects on food conversion efficiency among females during gestation or lactation.

F1 generation: Food conversion efficiency values were similar to controls for males and females given bicyclopyrone during pre-pairing.

Dose received
F0 and F1 generations: Dose rates (based on nominal dietary levels of bicyclopyrone were calculated in terms of mg bicyclopyrone/kg body weight. Mean values are shown below:

Table 10: Mean dose received (mg/kg bw/day)

	P generation			F1 generation		
bicyclopyrone (ppm)	**25 ppm**	**500 ppm**	**5000 ppm**	**25 ppm**	**500 ppm**	**5000 ppm**
Males	1.9 ± 0.5	38.4 ± 10.7	377.3 ± 98.6	2.4 ± 0.8	48.9 ± 16.1	493.8 ± 152.1
Females (Total)	2.5 ± 1.0	49.5 ± 18.1	501.1 ± 213.8	2.8 ± 1.2	55.9 ± 20.9	566.9 ± 200.7
Pre-pairing	2.1 ± 0.3	42.4 ± 6.7	409.6 ± 54.1	2.5 ± 0.6	49.9 ± 13.1	507.1 ± 122.0
Gestation	2.0 ± 0.1	40.4 ± 1.1	405.3 ± 9.7	2.1 ± 0.1	42.6 ± 0.6	438.7 ± 12.6
Lactation	4.3 ± 1.0	82.3 ± 16.2	902.0 ± 181.2	4.7 ± 1.3	87.3 ± 21.3	874.5 ± 198.8
Average of F0 and F1 Males*	2.15	43.65	435.55	NA	NA	NA
Average of F0 and F1 Females*	2.65	52.7	534	NA	NA	NA

Data were taken from pages 72-75 of the study report
*Calculated by the Health Effects Division

Reproductive function:
Oestrous cycle length and periodicity:
F0 and F1 generations: There was no effect of treatment on the mean number of oestrous cycles or the average length of oestrous cycles recorded during the pre-pairing period in F0 or F1 females.

Sperm measures:
F0 and F1 generations: There was no effect of treatment with bicyclopyrone on F0 generation sperm parameters. In the F1 there was an increase in the number of abnormal sperm and a decrease in sperm velocities for males given 5000 ppm. The decreases at 5000 ppm were as follows all in the F1 generation; velocity of actual path (↓9%), straight line velocity (↓13%) and miscellaneous abnormalities (↑117%).

Table 11: Summary of semen analysis and sperm morphology

Parameter	Dietary concentration of bicyclopyrone (ppm)							
	P Males				F1 Males			
	0	**25**	**500**	**5000**	**0**	**25**	**500**	**5000**
Number	25	25	24	25	24	25	25	24

Motility %	87.9 ± 3.9	86.3 ± 3.9	83.4 ± 10.4	84.8 ± 5.9	61.0 ± 9.0	61.6 ± 9.1	61.2 ± 9.9	62.8 ± 8.4
VAP µm/s	127.4 ± 10.2	130.5 ± 11.7	133.0 ± 10.4	131.6 ± 10.1	99.5 ± 10.3	92.6 ± 12.3	92.1 ± 11.3	91.0* ± 10.1 (↓9%)
VSL µm/s	77.3 ± 7.5	76.0 ± 6.5	74.3± 5.3	75.5 ± 5.3	49.7 ± 9.0	45.9 ± 10.5	44.9 ± 9.5	43.0* ± 7.7 (↓13%)
No normal	197 ± 2	197 ± 2	195 ± 6	195 ± 3	193 ± 3	192 ± 3	192 ± 2	189*** ± 5 (↓2%)
Misc abn	0.64 ± 0.57	0.68 ± 1.03	1.08 ± 1.21	0.48 ± 0.82	2.83± 2.24	3.68 ± 2.30	3.44 ± 2.24	6.13** ± 4.48 (↑117%)

VAP - velocity of actual path
VSL - straight line velocity
*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01
***statistically significantly different from control, p=<0.001
Misc. abn. - miscellaneous abnormalities (i.e. NOT headless, tail-less or reduced hook)

Reproductive Performance:

F0 generations: There was no effect of treatment on the time taken to mate; all paired animals mated and all females mated within one oestrous cycle. There was no effect on the fertility of either the males or females or on the mating activity of the animals.

There was no effect of treatment on the mean duration of gestation and, with the exception of one female given 5000 ppm, all pregnant females gave birth to live litters.

There was no effect of treatment on the mean number of pups born or upon the survival of the offspring to weaning. There was no effect of treatment on the number of implantation scars or upon the extent of post-implantation losses. There was no effect of treatment on sex ratio. The majority of females were in di-oestrus on Day 21 of lactation.

F1 generation: There was no effect of treatment on the mean duration of gestation and all pregnant females gave birth to live litters. There was no effect of treatment on the mean number of pups born or upon the survival of the offspring up to weaning. Overall pup survival for animals given 500 ppm was statistically significantly lower compared with controls. In the absence of a similar effect at 5000 ppm this was considered not to be related to treatment with bicyclopyrone.

There was no effect of treatment on the number of implantation scars or the extent of post implantation losses. There was no effect of treatment on sex ratios. The majority of females were in di-oestrus on Day 21 of lactation.

Table 12: Reproductive performance

Observation	Dietary concentration of bicyclopyrone (ppm)			
	0	**25**	**500**	**5000**
	P generation			
Mean precoital interval (days)	3.4 ± 0.6	2.7 ± 0.8	2.8 ± 1.1	2.5** ± 1.2 (↓26%)
Number paired	25	25	25	25
	males			
Copulation index %	100	100	100	100
Fertility index %	96	100	96	100
	females			
Copulation index %	100	100	100	100
Fertility index %	96	100	96	100
Number littered (Fertile)	24	25	24	25
Gestational index %	100	100	100	100
Mean duration of gestation (days)	22.5 ± 0.6	22.6± 0.5	22.8 ± 0.4	22.8 ± 0.4
	0	**25**	**500**	**5000**
	F1 generation			
Mean precoital interval (days)	3.0 ± 1.3	2.9 ± 1.5	2.8 ± 1.1	3.2 ± 3.2
Number paired	25	25	25	25
	males			
Copulation index %	92	100	100	100
Fertility index %	95.7	92.0	92.0	92.0
	females			
Copulation index %	92.0	100	100	100
Fertility index %	95.7	92	92	92
Number littered	22	23	23	23
Gestational index %	100	100	100	100
Mean duration of gestation (days)	22.6 ± 0.6	22.5 ± 0.5	22.7 ± 0.5	22.8 ± 0.4

Data were taken from pages 99-102, and 170-174 of the study report
**statistically significantly different from control, p=<0.01

Sacrifice and pathology:
Parental animals:
Macroscopic findings: Macroscopic changes are listed in table 13.

F0 and F1 generations: Treatment-related changes in the eyes (including opaque colour) were seen in both F0 and F1 generations, predominantly in males in the 5000 ppm group (72 and 36%, respectively). These changes were seen in all treatment groups for the F0 and F1 males (≥24% of animals per group).

In the F0 generation, there was an increased incidence of kidney pelvic dilatation recorded in the male kidneys of all treated groups (16-24%). Enlarged spleens were observed in the F0 generation, high dose females.

Pelvic dilatation was also seen in the kidneys in all F1 generation groups with an increased incidence in the treated groups (32-64% for F1 males and 12-48% for F1 females).

Table 13: Intergroup comparison of macroscopic findings – parents

Finding	Dietary concentration of bicyclopyrone (ppm)							
	Males				Females			
	P generation							
	0	25	500	5000	0	25	500	5000
Number examined	25	25	25	25	25	25	25	25
Eyes: opaque/pale colour and/or red striations	0	6 (24%)	14 (56%)	18 (72%)	0	0	2 (8%)	2 (8%)
Kidney: pelvic dilatation	1	4 (16%)	5 (20%)	6 (24%)	0	0	0	0
	F1 generation							
Number examined	25	25	25	25	25	25	25	25
Eyes: opaque/pale colour and/or red striations	0	13 (52%)	10 (40%)	9 (36%)	0	1 (4%)	6 (24%)	6 (24%)
Kidney: pelvic dilatation	3 (12%)	8 (32%)	16 (64%)	10 (40%)	1 (4%)	3 (12%)	10 (40%)	12 (48%)

Data were taken from pages 116-123, and 190-197 of the study report

Organ weights:

F0 generation: Statistically significant increases in adjusted liver, kidney and thyroid weights were observed in F0 males in the 25, 500 and 5000 ppm groups. There was no clear dose response for these increases. Statistically significant increases in adjusted liver weights were also seen at all dose levels in F0 females. Kidney weights in F0 females were increased at 5000 ppm but not at 25 or 500 ppm. Thyroid weights were not increased in females.

F0 Females given 5000 ppm showed a small increase in spleen weight compared with controls, but there was no similar effect at lower doses, in F1 females or in F0 parental males. In the absence of any similar findings in F0 males or in F1 parental animals the finding in 5000 ppm F0 females is considered to be incidental to treatment.

F1 generation: Statistically significant increases in the adjusted liver, kidney and thyroid weights were observed in F1 males in the 25, 500 and 5000 ppm groups. There was no clear dose response for these increases. Statistically significant increases in adjusted liver weights were observed at 5000 ppm in F1 females. Kidney weights in F1 females were increased at 5000 ppm but not at 25 or 500 ppm. Thyroid weights were not increased in females.

In F1 males and females, adjusted brain weights were statistically significantly lower than controls at 500 and 5000 ppm. As there were no decreases in absolute brain weights, these differences were considered to be secondary to lower bodyweight and slower growth in pups at these dose levels.

Microscopic findings:

F0 generation: Treatment-related centrilobular hepatocyte hypertrophy was seen in the livers of F0 males at all dose levels. In the vagina of F0 females receiving 5000 ppm there was an increased incidence of lactational dioestrus and presence of anoestrus. This is considered to be a result of prolonged suckling of the lower weight pups in this group.

Histopathological findings in the thyroid glands of F0 parental males and females at all dose levels were consistent with increased colloid turnover and were considered not to be of toxicological significance.

F1 generation: Treatment-related centrilobular hepatocyte hypertrophy was seen in the livers of F1 males at all dose levels. In F1 animals only there was an increased incidence and severity of renal pelvic dilatation in the 500 ppm and 5000 ppm dose groups. In the vagina of F1 females receiving 5000 ppm there was an increased incidence of lactational dioestrus and presence of anoestrus. This is considered to be a result of prolonged suckling of the lower weight pups in this group.

Histopathological findings in the thyroid glands of F1 parental males and females at all dose levels were consistent with increased colloid turnover and were considered not to be of toxicological significance.

The number of follicles in the ovaries of F1 females was similar across all the groups, including controls.

Offspring:
Viability and clinical signs:
F1 generation: There were 3 total litter losses for the F0 generation; 1 at 500 ppm and 2 at 5000 ppm. There were no pup clinical signs or mortalities that were considered to be related to treatment with the test article.

F2 generation: There were 4 total litter losses for the F1 generation; 1, 2 and 1 litter losses for animals given 25, 500 and 5000 ppm respectively. There were no pup clinical signs or mortalities that were considered to be related to treatment with bicyclopyrone.

Table 14: Litter parameters for F1 and F2

Observation	bicyclopyrone dose group (ppm)			
	0	25	500	5000
	F1 litters			
Mean number of pups born	11.2 ± 2.5	11.6 ± 2.2	10.5 ± 2.7	10.2 ± 2.5
Mean live birth index %	99.7	98.3	95.6	94.9
Mean viability index (day 4) % (a)	96.1	94.3	90.0	89.7
Mean viability index (day 7) % (b)	94.7	92.5	92.3 (23)	96.1 (22)
Mean viability index (day 14) % (c)	85.5	89.0	96.4 (23)*	95.3 (22)*
Mean lactation index %	80.8	84.2	89.0 (23)	92.1 (22)
Mean cumulative survival index %	77.9	78.2	80.1	81.1
% males at birth	51.5	51.7	49.8	45.8
	F2 litters			
Mean number of pups born	10.6 ± 2.8	11.1 ± 1.6	11.2 ± 2.2	9.7 ± 3.8
Mean live birth index %	99.4	98.4	98.8	99.4
Mean viability index (day 4) % (a)	98.0	93.6	86.6	93.1
Mean viability index (day 7) % (b)	98.4	100 (22)	94.7 (22)	95.7
Mean viability index (day 14) % (c)	93.8	97.7 (22)	86.1 (22)	96.7 (22)
Mean lactation index %	92.4	97.7 (22)	87.9 (22)	95.1 (22)
Mean cumulative survival index %	90.2	91.2	71.5*	85.5
% males at birth	47.0	49.7	51.7	46.9

Data were taken from pages 102 and 174 of the study report
*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01

Body weight:
F1 generation: Absolute body weights in females was lower than controls at 5000 ppm on day 21 (↓13%). Body weight gains to day 17 of female pups at 5000 ppm (↓11%), and to day 21 at 500 and 5000 ppm were lower than controls (↓11 and 17%, respectively). Absolute body weights at day 21 and body weight gains to day 21 of male pups given 500 or 5000 ppm were lower than controls. There was no effect on pup body weight following treatment at a dose level of 25 ppm.

F2 generation: Absolute body weights on day 1 of male and female pups given bicyclopyrone were similar to controls. However, body weight gains from day 1 to 21 were statistically significantly lower at 5000 ppm (↓13% for males and ↓14% for females), and statistically significantly lower body weights on day 21 of lactation (↓12% for males and 13% for females). There was no effect on pup body weight or body weight gain at 500 or 25 ppm.

Table 15: Body weight for F1 and F2 pups

Observation	bicyclopyrone dose Group (ppm)			
	0	25	500	5000
	F1 litters			
Mean male pup body weight (day 1)	6.2 ± 0.4	6.3 ± 0.5	6.2 ± 0.6	6.3 ± 0.7
Mean male pup body weight (day 17)	31.9 ± 3.7	31.3 ± 3.7	29.6 ± 4.3	29.9 ± 3.5
Mean male pup body weight (day 21)	41.5 ± 4.9	40.8 ± 5.6	36.8* ± 5.9 (↓11%)	36.3** ± 5.6 (↓13%)
Mean male pup bw gain (days 1-21)	35.3 ± 4.7	34.5 ± 5.3	30.6** ± 5.6 (↓11%)	29.9** ± 5.3 (↓15%)
Mean female pup body weight (day 1)	5.9 ± 0.4	6.1 ± 0.6	5.9 ± 0.6	6.1 ± 0.7
Mean female pup body weight (day 17)	31.2 ± 3.9	30.2 ± 3.9	29.7 ± 3.9	28.6 ± 3.8
Mean female pup body weight (day 21)	40.4 ± 5.0	39.2 ± 5.5	36.6 ± 5.4	35.0** ± 6.0 (↓13%)
Mean female pup bw gain (days 1-21)	34.5 ± 4.9	33.0 ± 5.2	30.8* ± 5.4 (↓11%)	28.8** ± 6.0 (↓17%)
	F2 litters			
Mean male pup body weight (day 1)	6.5 ± 0.6	6.4 ± 0.6	6.1 ± 0.7	6.3 ± 0.8
Mean male pup body weight (day 17)	31.4 ± 4.8	29.6 ± 2.3	30.5 ± 3.9	29.9 ± 4.3
Mean male pup body weight (day 21)	41.4 ± 6.9	38.0 ± 4.3	38.1 ± 5.7	36.6* ± 6.0 (↓12%)
Mean male pup bw gain (days 1-21)	35.0 ± 6.6	31.6 ± 4.0	32.0 ± 5.2	30.3* ± 5.4 (↓13%)
Mean female pup body weight (day 1)	6.1 ± 0.6	6.0 ± 0.6	5.7 ± 0.8	5.9 ± 0.8
Mean female pup body weight (day 17)	30.4 ± 5.1	29.1 ± 2.9	29.1 ± 3.7	29.0 ± 4.0
Mean female pup body weight (day 21)	40.1 ± 7.3	37.4 ± 4.4	36.4 ± 5.3	35.0* ± 5.8 (↓13%)
Mean female pup bw gain (days 1-21)	34.0 ± 6.9	31.4 ± 4.0	30.6 ± 4.8	29.1* ± 5.3 (↓14%)

Data were taken from pages 104 and 176 of the study report
*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01

Ophthalmoscopy:

F1 generation: Treatment-related corneal opacity and roughness and vascular keratitis were seen in male and female pups given 500 or 5000 ppm bicyclopyrone. These changes were not observed in controls or pups given 25 ppm.

F2 generation: Treatment-related corneal opacity and roughness and vascular keratitis were seen in a dose related manner in male and female pups given 500 or 5000 ppm bicyclopyrone. These changes were not observed in control pups or pups given 25 ppm.

Table 16: Incidence of opacity and roughness and vascular keratitis in the F1 and F2 pups

	Dietary concentration of bicyclopyrone (ppm)			
	0	**25**	**500**	**5000**
	F1 pups			
Number of litters	24	25	23	22
Corneal opacity & roughness - males	0	0	8 (35%)	8 (36%)
Corneal opacity & roughness - females	0	0	10 (43%)	8 (36%)
Vascular keratitis – males	0	0	2 (9%)	4 (18%)
Vascular keratitis - females	0	0	4 (17%)	3 (14%)
	F2 pups			
Number of litters	22	22	21	22
Corneal opacity & roughness - males	0	0	11 (52%)	14 (64%)
Corneal opacity & roughness - females	0	0	8 (38%)	14 (64%)
Vascular keratitis – males	0	0	2 (10%)	11 (50%)
Vascular keratitis - females	0	0	3 (14%)	8 (36%)

Data were taken from pages 48 and 59 of the study report

Sexual maturation:

F1 generation: There was a statistically significant delay in attainment of preputial separation observed for males given 25, 500 or 5000 ppm, when compared with controls (↑5, 5 and 9%). As the magnitude of these increases was small, with no other associated delays in sexual development in male pups, the slight increase in preputial separation in treated F1 generation males is not considered to be of toxicological concern.

There was no effect of treatment on the day of vaginal perforation observed for females. Although females given 500 ppm showed a statistically significant later perforation, females given 5000 ppm were similar to controls.

Anogenital distance:

F2 generation: There was no effect of treatment with bicyclopyrone on the anogenital distance of male or female pups.

Table 17: Summary of sexual development in the F1 and F2 pups

	Dietary concentration of bicyclopyrone (ppm)			
	0	**25**	**500**	**5000**
	F1 pups			
Number examined	25	25	25	25
Day of preputial separation	45.3 ± 2.6	47.5** ± 2.8 (↑5%)	47.7** ± 2.0 (↑5%)	49.4** ± 3.1 (↑9%)
Weight at preputial separation (g)	178.9 ± 19.5	193.4 ± 19.6	183.3 ± 18.4	180.6 ± 14.6
	F2 pups			
Number examined	22	23	23	23
Anogenital distance - males	2.5 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	2.6 ± 0.4
Anogenital distance - females	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1

Data were taken from pages 146 and 175
*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01

Offspring *postmortem* results:

Necropsy findings:

F1 generation: At 5000 ppm, there was a slight increase in the incidence of no milk in the stomach of pups found dead or prematurely sacrificed, when compared with controls. There were no treatment-related abnormalities recorded at day 21.

F2 generation: There was a higher incidence of no milk in the stomach of pups found dead or prematurely sacrificed at 500 or 5000 ppm, compared with controls. There were no treatment-related abnormalities recorded at day 21.

Organ weights: Select absolute and adjusted organ weights are listed on table 18.

F1 generation: Decreases in in absolute liver weights were observed in male pups at 500 and 5000 ppm. Increases in adjusted liver weights were observed in F1 male and female pups at 5000 ppm and in females only at 500 ppm. Kidney weights of F1 females were increased in the 500 and 5000 ppm groups. F1 male and female brain weights were statistically significantly lower than controls at 500 and 5000 ppm. As substantial post-natal development occurs in rats it is likely that lower brain weights reflect delayed growth associated with decreased body weight gains in these treatment groups.

F2 generation: Increases in liver weights were observed in F2 male and female pups at 5000 ppm and in females only at 500 ppm. Kidney weights of F2 females were increased in the 500 and 5000 ppm groups and in F2 males at 5000 ppm group. F2 male and female brain weights were statistically significantly lower than controls at 500 and 5000 ppm. As substantial post-natal development occurs in rats it is likely that lower brain weights reflect delayed growth associated with decreased body weight gains in these treatment groups.

Table 18: Intergroup comparison of selected pup organ weights (g) – absolute and adjusted for final body weight

Organ	Dietary concentration of bicyclopyrone (ppm)							
	Males				Females			
	0	**25**	**500**	**5000**	**0**	**25**	**500**	**5000**
	F1 pups							

Liver (abs.)	1.80 ± 0.34	1.70 ± 0.34	1.48* ± 0.40 (↓18%)	1.52* ± 0.38 (↓16%)	1.69 ± 0.30	1.66 ± 0.28	1.53 ± 0.28	1.55 ± 0.35
Liver (adj.)	1.57	1.59	1.68	1.71* (↑9%)	1.53	1.57	1.66** (↑8%)	1.70*** (↑11%)
Kidneys (abs.)	0.533 ± 0.082	0.493 ± 0.076	0.440** ± 0.104 (↓18)	0.452** ± 0.081 (↓16)	0.526 ± 0.087	0.513 ± 0.072	0.473 ± 0.072	0.466 ± 0.102
Kidneys (adj.)	0.479	0.465	0.489	0.496	0.482	0.487	0.508* (↑5%)	0.510* (↑6%)
Brain (abs.)	1.38 ± 0.05	1.36 ± 0.07	1.25*** ± 0.11 (↓10%)	1.24*** ± 0.06 (↓10%)	1.34 ± 0.08	1.30 ± 0.06	1.22*** ± 0.05 (↓9%)	1.22*** ± 0.07 (↓9%)
Brain (adj.)	1.34	1.34	1.28*** (↓4%)	1.28*** (↓4%)	1.31	1.29	1.25*** (↑5%)	1.25*** (↑6%)
	F2 pups							
Liver (abs.)	1.70 ± 0.33	1.52 ± 0.25	1.55 ± 0.34	1.49 ± 0.35	1.58 ± 0.33	1.57 ± 0.30	1.58 ± 0.29	1.51 ± 0.33
Liver (adj.)	1.52	1.56	1.60	1.60* (↑5%)	1.47	1.53	1.61*** (↑10%)	1.62*** (↑10%)
Kidneys (abs.)	0.53 ± 0.10	0.46* ± 0.05	0.48 ± 0.09	0.48 ± 0.10	0.52 ± 0.10	0.48 ± 0.07	0.50 ± 0.08	0.50 ± 0.10
Kidneys (adj.)	0.48	0.47	0.50	0.51** (↑6%)	0.48	0.47	0.51* (↑6%)	0.53*** (↑10%)
Brain (abs.)	1.39 ± 0.05	1.35 ± 0.04	1.28*** ± 0.08 (↓8%)	1.25*** ± 0.07 (↓10%)	1.31 ± 0.08	1.33 ± 0.05	1.25** ± 0.06 (↓5%)	1.22*** ± 0.07 (↓7%)
Brain (adj.)	1.37	1.36	1.29*** (↓6%)	1.27*** (↓7%)	1.29	1.32	1.26* (↓2%)	1.24*** (↓4%)

Data taken from pages 143 to 145 and 219 to 222 of the study report (standard deviations not calculated for adjusted organ weights)
*statistically significantly different from control, p=<0.05
**statistically significantly different from control, p=<0.01
***statistically significantly different from control, p=<0.001

INVESTIGATOR'S CONCLUSIONS: The Low Observed Adverse Effect Level (LOAEL) for systemic toxicity in parental animals is 25 ppm (1.9-2.4 mg/kg bw/day in males and 2.5-2.8 mg/kg bw/day in females) based on ocular effects.

The No Observed Adverse Effect Level (NOAEL) for systemic toxicity and sexual development in pups was 25 ppm.

REVIEWER'S COMMENTS:

Based upon these effects, the parental LOAEL is 25 ppm (2.15/2.65 [M/F]) based upon ocular effects (corneal opacity and vascular keratitis in P and F1 males) and an increased incidence of pelvic dilation of the kidney (P and F1 males, and F1 females). The parental NOAEL was not established.

Based upon these effects, the offspring LOAEL is 500 ppm (43.65/52.7 mg/kg/day [M/F]) based upon decreased absolute body weights and body weight gains in the F1 generation, and an increased incidence of litters with ocular effects (corneal opacity and roughness, and

vascular keratitis) in the F1 and F2 generations. The offspring NOAEL is 25 ppm (2.15/2.65 mg/kg/day [M/F]).

Based upon these effects, the reproductive LOAEL is 5000 ppm (435.5/534 mg/kg/day [M/F]) based upon changes in sperm parameters in the F2 generation, and a decreased precoital interval and an increased time for preputial separation in the F1 generation. The reproductive NOAEL is 500 ppm (43.65/52.7, mg/kg/day [M/F]).

Bicyclopyrone is not considered a reproductive toxicant in the rat. APVMA/OCS (Australia) believes that the decrease in the precoital interval should not be considered a toxicologically adverse finding, and should not be a part of the LOAEL.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the need for a two-generation reproduction study OPPTS 870.3800 [§83-4]; OECD 416 in the rat. EPA and PMRA (Canada), agree on the regulatory decision for this study. All three agencies agree on the regulatory classification for this study.

(Davies S and Penn L, 2012)

Appendix

STUDY TYPE: Preliminary Multigeneration Reproduction Study (rat) No applicable guidelines

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Davies S and Penn L, 2009. NOA449280: Oral (dietary) multigeneration range finding study in the rat. Sequani Ltd., Ledbury, UK. Laboratory Report No. BFI0003, 30 October 2009. Unpublished. (Syngenta File No.NOA449280/11055). MRID 47841991

SPONSOR: Syngenta Limited, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

EXECUTIVE SUMMARY

In a multigeneration dose range-finding study, bicyclopyrone (NOA449280, purity 94.5%) was administered to young adult, Crl:WI (Han) rats. Four groups of 12 males and 12 females received NOA449280 in the diet for 10 weeks and were then paired (one male with one female) for mating, with a control group of 12 males and 12 females receiving the diet only. Formulated diet was available for the males throughout the mating period until necropsy and to females during the mating period, gestation and lactation and up to termination at Day 22 of age of the F1 generation. The animals were provided with diet containing the test article at dose levels of 0, 25, 500, 2500 or 5000 ppm. The amount of compound received (mg/kg/day) was not calculated by the registrant. The time of onset and completion of parturition were recorded. The females were allowed to rear their offspring to weaning on Day 22 of lactation and nursing and nesting behaviour of the maternal animals was observed. All animals were examined for effects on general condition, body weight and food consumption. Ophthalmoscopy examinations were performed for all F0 animals before and after pairing.

The males were killed once successful littering was completed and the females were killed on Day 22 of lactation and all were given a macroscopic examination *post mortem.* For all F0 animals, terminal body weight, liver and kidney weights were recorded and liver and kidneys were retained for possible subsequent examination. Before discarding the uterus, the implantation scars in each uterine horn were counted for all mated F0 females. For any apparently non-pregnant F0 female the uteri were stained with ammonium sulphide for confirmation of pregnancy status.

The total litter size was recorded after the completion of littering and litter size was recorded daily thereafter and pups were sexed on Days 1, 4, 7, 14, 21 and 22 of lactation. All pups were examined after the completion of littering for malformations and the pups were examined daily for clinical signs of toxicity and weighed on Days 1, 4, 7, 14 and 21. Where possible, one pup/sex/group was given an ophthalmoscopy examination between Days 19 and 22. A necropsy was performed on any pups that died or were sacrificed prematurely during lactation. Surviving F1 pups were killed on Day 22 of lactation. Three pups/sex/group,

where possible, were examined. Terminal body weight and the weights of the liver and kidneys from one pup/sex/litter were recorded and these pups were given a macroscopic examination. Liver and kidneys together with any grossly abnormal tissue were retained for possible subsequent examination.

At 25 ppm in the parental males, there was an increased incidence of vascular corneal opacity (33%). For the parental males, there was a treatment related increase in adjusted liver and kidney weights (↑19 and ↑9%, respectively).

At 500 ppm in both the parental males and females, there was an increased incidence of corneal opacity in males and females (66 and 75%, respectively). For the parental males, there was a treatment related increase in adjusted liver and kidney weights (↑27 and ↑13%, respectively).

At 2500 ppm in both the parental males and females, there was an increased incidence of corneal opacity in males and females (58 and 83%, respectively). In addition there was a statistically significant decrease in body weight gain Days 1-14 of lactation for females receiving 2500 ppm (↓34%). For the parental males, there was a treatment related increase in adjusted liver and kidney weights (↑20 and ↑10%, respectively).

At 5000 ppm in both the parental males and females, there was an increased incidence of corneal opacity in males and females (92 and 68%, respectively). Lower body weight gains were apparent for males receiving 5000 ppm for the first week of the pre-mating period only (↓16%). Lower body weights and body weight gains were evident in females receiving 5000 ppm during the pre-mating (↓19-35%), gestation (↓17-22%), and lactation periods (↓32%). In addition there was a statistically significant decrease in body weight gain Days 1-14 of lactation for females receiving 5000 ppm (↓32%). Food consumption of F0 females at 5000 ppm was lower than controls throughout lactation (↓16-23%). For parental males there was an increase in the adjusted liver and kidney weights (↑22% and ↑16%, respectively).

Based upon these effects, the LOAEL for parental males is 25 ppm based upon ocular effects. The parental NOAEL for males was not established. The LOAEL for parental females is 500 ppm based upon ocular effects. The NOAEL for parental females is 25 ppm.

Corneal opacities were observed in F1 pups in the groups receiving 500, 2500 and 5000 ppm. There was no effect of treatment on the pup survival indices or sex ratio, when compared with controls. The percentage of pups surviving to Day 22 was lower than expected (70-76%) in all groups. Body weights on Day 21 of lactation were lower than controls for male pups receiving 5000 ppm. At necropsy, early decedent pups from groups given 500, 2500 or 5000 ppm were found to have an increased incidence of no milk in the stomach.

Based upon these effects, the offspring NOAEL is 25 ppm based upon increased corneal effects at the LOAEL of 500 ppm.

There was no effect of treatment on the time taken to mate, fertility, the duration of gestation or the proportion of pups born live. Females given 2500 or 5000 ppm had lower statistically significant mean numbers of pups on Day 1 (9.6 and 9.8, respectively), when compared with Controls (11.9). There was a corresponding lower number of uterine implantation scars in females given 5000 ppm (↓24%).

The reproductive LOAEL is 2500 ppm based upon lower litter sizes and mean number of uterine implantation sites. The reproductive NOAEL is 500 ppm.

A high dose of 5000 ppm and a low dose of 25 ppm were considered to be appropriate dose levels for the subsequent multi generation study in the rat.

This study is classified as totally reliable (**acceptable/non-guideline study)** and satisfies the need for a two-generation reproduction range-finding study in the rat.

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anw Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Chronic Toxicity (oral (capsule), dog) OECD Guidelines No. 452 (1981) EPA OPPTS 870.4100 (1998) Directive 88/303/EEC (OJL 133 1988) B.30

TEST MATERIAL (PURITY): NOA449280 (94.5% w/w)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Braun L, 2010. NOA449280 – 52-Week Oral (Capsule) Toxicity Study in the Dog. Harlan Laboratories Ltd., (former RCC Ltd), Zelgliweg 1, 4452 Itingen / Switzerland. Laboratory Report No. B69737. 24 September 2010. Unpublished. (Syngenta File No. NOA449280_11115) MRID 47841977

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of this study.

Justification for Test System Selection: The dog is the preferred non-rodent species for regulatory studies and the Beagle was used because of the substantial background data available for this breed, in this Laboratory, relating to studies of this type. The oral route (via gelatin capsules) was chosen for administration of NOA449280, as this represents a possible route of exposure in humans and other mammalian species.

EXECUTIVE SUMMARY

In a chronic toxicity study in dogs (MRID #47841977), groups of four male and four female Beagle dogs were dosed orally by capsule with bicyclopyrone at 0, 2.5, 25 or 125 mg/kg/day for a period of up to 52 weeks. Clinical signs, body weight and food consumption were recorded throughout the study. Ophthalmoscopy and veterinary examinations were performed and blood and urine samples were collected for clinical laboratory investigations at intervals during the study. In addition, blood samples were collected for analysis of plasma bicyclopyrone, metabolite CSAA915194 and tyrosine levels. Following completion of the scheduled treatment

period, a detailed necropsy was performed on all animals and selected organs were weighed. A full set of tissues and organs were prepared and examined histopathologically

There were no differences observed during veterinary examinations, or in body weights, food consumption, hematology or urinalysis parameters, organ weights or macroscopic findings that were considered to be related to treatment with the test item.

At 2.5 mg/kg/day, there was minimal to moderate chromatolysis and swelling of selected neurons in the dorsal root ganglia in two males and three females at 2.5 mg/kg/day (50% and 75%) compared to 0% in the controls. There was also minimal degeneration of nerve fibers of the sciatic nerve in two males (50%) and one female (25%) compared to 25% in the controls for males and 0% in females.

At 25 mg/kg/day there were persistent corneal opacities which developed over the course of treatment in two males and one female. One male showed sensitivity to light during the examination. There was a decrease in total bilirubin at week 52 (↓42%) and cholesterol levels at week 13 (↓26%) in females. In the absence of any effects on other biochemical parameters or any histopathological changes in the liver this finding is considered to be of low toxicological significance. There was minimal to moderate chromatolysis and swelling of selected neurons in the dorsal root ganglia in both sexes (100%). There was also minimal degeneration of nerve fibers of the sciatic nerve in two males (50%) and one female (25%) compared to 25% in the controls for males and 0% in females.

At 125 mg/kg/day, there were persistent corneal opacities which developed over the course of treatment in one female. One female showed sensitivity to light during the examination. One male was found dead on day 336 after one day of low food consumption but a cause of death could not be determined. There were decreased cholesterol levels at weeks 13, 26 and 52 (↓53-57%) in females, and at weeks 26 and 52 (↓52-53%) in males. In the absence of any effects on other biochemical parameters or any histopathological changes in the liver this finding is considered to be of low toxicological significance. There was minimal to moderate chromatolysis and swelling of selected neurons in the dorsal root ganglia in males and females (75 and 50%). There was also minimal degeneration of nerve fibers of the sciatic nerve in two males (50%) and two female (50%) compared to 25% in the controls for males and 0% in females.

Regarding the toxicokinetic analysis, exposure was primarily to the parent compound. The metabolite reached only 0.8 to 5.4% of the AUCτ of bicyclopyrone. With a 50-fold dose range investigated (2.5-50 mg/kg/day), exposure to bicyclopyrone increased with AUCτ ratios of 15 to 42. For CSAA915194, AUCτ increased in males with ratios of 23 to 52 and in females with ratios of 41 to 55. Following treatment with bicyclopyrone at 2.5 mg/kg/day, tyrosine AUCτ increased by a factor of 6.2 to 22 when compared to controls. No further increase in tyrosine AUCτ (ratios: 0.9 to 1.3) was found in dogs receiving doses of 25 and 125 mg/kg/day. There was no consistent gender difference throughout all treated groups and at all sampling occasions for parent compound, metabolite and tyrosine; except at 2.5 mg/kg/day, where exposure to CSAA915194 was lower in females than in males. At each bicyclopyrone treatment level,

exposure to parent compound, metabolite and tyrosine remained similar after 4, 13, 26 and 52 weeks of repeated dosing.

Based on the results of this study, the LOAEL is 2.5 mg/kg/day based upon an increased incidence of chromatolysis and swelling of selected neurons in the dorsal root ganglia and degeneration of nerve fibers in the sciatic nerve roots in both sexes at all treatment levels. A NOAEL was not observed.

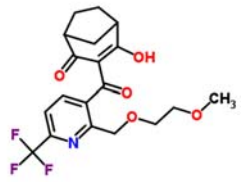
This study is classified as totally reliable (**acceptable/guideline)** and satisfies the guideline requirements for a chronic oral study [OPPTS 870.4100, OECD 452] in dogs.

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, Flagging and Quality Assurance statements were provided.

MATERIALS AND METHODS

Materials:

Test material:	Bicyclopyrone (NOA449280)
Description:	Beige brown powder
Lot/Batch number:	SEZ3AP006/MILLED
Purity:	94.5% w/w
CAS#:	Not stated
Stability of test compound:	Expiry date (reanalysis date): Mar-2011



Vehicle: None, the test substance was administered as supplied via gelatine capsules.

Test animals:	
Species:	Dog
Strain:	Pure-bred Beagle
Age/weight at dosing:	6-7 months, 7.0-10.2 kg
Source:	Harlan Laboratories Ltd. Laboratory Animal Services 4414 Füllinsdorf / Switzerland
Housing:	Animals were housed either individually or in groups of two/group/sex, in solid floor pens with minimum of 2.0 square meters of floor space per dog.
Acclimatization period:	28 days
Diet:	Animals were offered 350 ± 1 g (up to week 11) and 400 ± 1 g (from week 12) pelleted standard Kliba 3353 dog maintenance diet (Provimi Kliba AG, 4303 Kaiseraugst / Switzerland)
Water:	Community tap water was supplied *ad libitum* by an automatic watering system.
Environmental conditions:	Temperature: 20 ± 3°C Humidity: 30-70% Air changes: Approximately 10-15 per hour Photoperiod: 12 hours light/12 hours dark

Study dates and Methods:

In-life dates: Start: 04 March 2008 End: 17/18 march 2009

Route and duration of administration: In a chronic toxicity study, groups of four male and four female Beagle dogs were dosed orally by capsule with bicyclopyrone at 2.5, 25 or 125 mg/kg/day for a period of up to 52 weeks. The control group received empty capsules.

Animal assignment: After arrival the animals were weighed and the weights ranked. Animals were allocated to groups based on a four by four Latin square. The allocation was checked for the presence of litter mates which were distributed throughout the groups. Thereafter, pre-treatment investigations were carried out on all dogs. Shortly before the start of treatment, the data obtained were reviewed and no adjustment considered necessary.

The group identification and animal numbers assigned to treatment are stated in the following table:

Table 1: Study Design

Test group	Dose level (mg/kg bw/day)	# male	# female
Control	0	1 - 4	17 - 20
Low	2.5	5 - 8	21 - 24
Medium	25	9 - 12	25 - 28
High	125	13 - 16	29 - 32

Table was taken from page 19 of the study report

Capsule preparation: The appropriate amount of test item (in terms of material as supplied) was weighed directly into gelatin capsules. The individual weights of test item required for daily administration were adjusted based on the most recently recorded body weight. The test item was administered to animal no. 31 in Size 12 (batch 1508) gelatin capsules from week 8 onwards due to gingival/teeth infections. Capsules were prepared up to seven days in advance and stored at room temperature (20 ± 5 °C), protected from direct sunlight.

The control animals received empty gelatin capsules of the same size (capsule size 11) and number as those given the high dose level animals.

Observations: Observations for viability were recorded at least twice daily from commencement of the pretest period.

Each animal was examined at least twice daily from commencement of the pretest period for any change in behaviour, reaction to treatment or ill-health. A description of any abnormality was recorded. Thorough examinations outside the pen were performed once weekly except in the first pre-test week due to a technical error. In addition, single animals were examined by the study veterinarian during the study and treated.

Bodyweight: The body weight of each animal was recorded at least once weekly from the pretest period and before necropsy.

Food consumption: Food consumption was recorded daily from commencement of the pretest period. The daily ration was weighed before and after feeding. Weekly mean food consumption was presented in the report and the individual daily values retained in the study raw data.

Ophthalmoscopic examination: Each animal was examined at pretest, week 13, week 26 and week 52 for abnormalities of the eyes at least 20 minutes after instillation of 0.5% tropicamide solution using a binocular indirect ophthalmoscope and the results were recorded. Unless otherwise indicated in the table, the contralateral eye was without abnormalities. The observation area included the cornea, conjunctiva, sclera, iris, lens and fundus.

Veterinary examination: Veterinary examinations were performed on all animals at pretest and during week 52. The examinations include spinal reflex (patellar reflex) and cranial nerves (pupil reflex, corneal reflex) as well as pulmonary and cardiac auscultation.

Haematology: Blood samples were collected from all animals during the pretest, week 13, week 26 and week 52.The samples were collected from the first animal of each group in the order 4, 1, 3, 2 followed by the second animal in this group order until all animals had been sampled. The dogs were fasted overnight but allowed access to water *ad libitum*. The samples were collected early in the working day to reduce biological variation caused by circadian rhythms. Blood samples were drawn from the jugular vein.

Anticoagulants used for blood collection were tri-potassium EDTA (hematology) or sodium citrate, 3.2% at a 9:1 ratio of blood to anticoagulant (coagulation). The following hematology parameters were determined:

Erythrocyte count	Platelet count
Hemoglobin concentration	Reticulocyte count
Hematocrit	Reticulocyte maturity index
Mean corpuscular volume	Total leukocyte count
Red cell volume distribution width	Differential leukocyte count
Mean corpuscular hemoglobin	Coagulation:
Mean corpuscular hemoglobin concentration	Thromboplastin time
Hemoglobin concentration distribution width	Activated partial thromboplastin time

Clinical chemistry: The anticoagulant used for blood collection was lithium heparin. The following clinical biochemistry parameters were determined:

Glucose	Alkaline phosphatase
Urea nitrogen	Gamma-glutamyl-transferase
Creatinine	Calcium
Bilirubin, total	Phosphorus
Cholesterol, total	Sodium
Triglycerides	Potassium
Aspartate aminotransferase	Chloride
Alanine aminotransferase	Protein, total
Glutamate dehydrogenase	Albumin
Creatine kinase	Globulin
Lactate dehydrogenase	Albumin/Globulin Ratio

Urinalysis: Urine samples were collected from all animals during the pretest, week 13, week 26 and week 52. The dogs were fasted overnight but allowed access to water *ad libitum*. Urine was collected into a specimen vial using a catheter.

The following parameters were determined:

Volume (catheterized sample)	Glucose
Specific gravity	Ketone
Colour	Urobilinogen
Appearance	Bilirubin
pH	Erythrocytes
Nitrite	Leukocytes
Protein	

Analysis of Plasma bicyclopyrone, CSAA915194 and Tyrosine Levels

Blood sampling: Blood samples were collected from all animals for analyses of bicyclopyrone, CSAA915194 and tyrosine in plasma. The animals were fasted overnight but allowed access to water *ad libitum*. During pretest (17-Mar-2008) one sample was taken from each animal early in the morning. During the treatment period the samples were collected in week 4 (10-Apr-2008), week 13 (13-Jun-2008), week 26 (12-Sep-2008) and week 52 (13-Mar-2009) from all animals before dosing and at 1, 2, 3, 4, 8, 12 and 24 hours after dosing.

On each occasion, approximately 5 mL of blood was drawn from the jugular vein and collected into lithium heparin blood collection tubes. Following centrifugation (1000g at 2 to 8 °C for 10 minutes), plasma was divided into two equal parts, transferred into plastic (polypropylene) tubes and placed on dry ice until storage. The samples collected during pretest and week 4 were stored at -20 ± 5 °C in the dark and then transferred to -80 ± 10 °C from week 5 onwards. The samples collected during weeks 13, 26 and 52 were stored at -80 ± 10 °C in the dark. For all samples, both aliquots were transferred on dry ice to the study scientist responsible for the bioanalysis determinations of bicyclopyrone, its metabolite CSAA915194 and tyrosine concentration in plasma.

Determination of bicyclopyrone, CSAA915194 and tyrosine concentration in dog plasma: The samples were analyzed using a sample preparation technique and a LC/MS/MS (liquid chromatography coupled with tandem mass spectrometric detection) method, formally validated according to GLP guidelines within Harlan Laboratories Studies B95220 (NOA449280 and CSAA915194) and B95231 (Tyrosine).

Toxicokinetic evaluation: The toxicokinetic evaluations were performed and reported using the validated software WinNonLin Version 5.2.1 (Pharsight Corporation, Mountain View, California 94041/USA). Toxicokinetic and statistical analysis appropriate to the data were performed.

Investigations *post mortem:*

All animals were anaesthetised by intravenous injection of sodium pentobarbital and killed by exsanguination at the end of the treatment period.

Macroscopic examination: All animals were examined *post mortem*. This involved an external observation and an internal examination of all organs and structures.

Organ weights: From all animals surviving to schedules termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

Adrenal gland (l, r)	Brain (including brainstem)
Epididymides (l, r)	Heart
Kidney (l, r)	Liver with gall bladder
Ovaries (l, r)	Spleen
Testes (l, r)	Thymus
Thyroid gland with parathyroid (l, r)	Uterus

Paired organs were weighed separately.

Tissue submission: The following tissues were examined *in situ*, removed and examined and fixed in an appropriate fixative. Bone marrow smears from the sternum and all animals were taken for possible further investigation.

Adrenal glands	Peyer's patches
Aorta	Pharynx[1]
Bone - sternum, femur including articular surface[1]	Pituitary gland
Bone marrow – femur, sternum	Prostate gland (accessory sex organ)
Brain - including sections of brainstem, medulla/pons, cerebrum and cerebellum	Salivary glands - mandibular, parotid, sublingual
Epididymides (fixed in Bouin's solution)	Sciatic nerve (in close proximity to the muscle)
Esophagus	Skeletal muscle - semimembranosus, tibialis cranialis, vastus medialis and gastrocnemius
Eyes with optic nerve (fixed in Davidson solution)	Skin and subcutaneous tissue
Gallbladder	Small intestine – duodenum, jejunum, ileum
Heart	Spinal cord – cervical (C1), midthoracic (T7) and lumbar (L7) segments including roots and dorsal root ganglia at lumbar levels
Kidneys and ureters	Spleen
Large intestine - cecum, colon, rectum	Stomach
Larynx[1]	Testes (fixed in Bouin's solution)
Liver	Thymus
Lungs with bronchi and bronchioles, infused with formalin	Thyroid gland
Lymph nodes - retropharyngeal, mesenteric	Tongue
Mammary glands (females only)	Trachea
Nasal cavities (only level 3 of 4)[1]	Urinary bladder
Ovaries	Uterus with cervix and oviducts
Pancreas	Vagina
Parathyroid gland	All gross lesions

[1] Marked tissues were collected and stored but not further processed or examined microscopically.

Microscopic examination: All selected organs and tissue samples were processed, embedded ad cut at nominal thickness of 4 micrometers, stained with haematoxylin and eosin and examined by light microscopy.

Statistics: Statistical tests were performed using appropriate computing devices or programs (SAS 9.2 TS Level 1M0). The following statistical approaches were used in this study:

All analyses were two-tailed for significance levels of 5% and 1%.

All means were presented with standard deviations.

If the variances were clearly heterogeneous, appropriate transformations (e.g. log, square root, double arcsine) were used in an attempt to stabilise the variances.

For continuous data body weights, cumulative body weight gain, food consumption, haematology, clinical biochemistry, quantitative urinalysis values (e.g. specific gravity) and absolute organ weights were analyzed initially by a one-way analysis of variance (ANOVA).

Organ weights were also analyzed by analysis of covariance (ANCOVA) on final body weight (Shirley, 1977). This statistical analysis provided an adjusted organ weight value, which was displayed in the results table along with flags for statistical significance.

Summary values of organ to body weight ratios were presented but were not analysed statistically.

For all of the parameters evaluated initially by ANOVA or ANCOVA, Dunnett's test was used to compare the control and treated groups, based on the error mean square in the ANOVA or ANCOVA. The Dunnett's test was performed for all continuous data parameters, regardless of whether the initial ANOVA or ANCOVA was statistically significant, and statistical flags were presented in the tables of results.

Macropathology and micropathology incidence data were analyzed using Fisher's Exact Test.

Qualitative parameters (e.g. possible values of 0, 1, 2 or present/absent) not specifically mentioned above that yield qualitative data were presented as summary data, but were not analyzed statistically.

Individual values were rounded before printing. All derived values that appeared in the report tables represented the rounded results of calculations that were based on the exact (non-rounded) raw data values. Statistical analyses also were carried out on the exact raw data values.

RESULTS AND DISCUSSION

Viability/Mortality: One male, no. 15, dosed at 125 mg/kg/day was found dead on Day 336 following one day of low food consumption. There were no clinical signs observed before the death which would indicate morbidity.

All remaining animals survived the scheduled treatment period.

Clinical observations: With the exception of an opacity in the left eye of one high dose female, there were no adverse clinical signs considered to be related to treatment with the test item.

Loose or watery faeces were recorded in several animals of all groups, including controls, and were therefore considered not to be related to treatment with the test item. Mucus, dark or red faeces, salivation and vomiting of mucus, discoloured white or yellow mucus, feed or capsule were generally recorded on single occasions in single animals. The incidences of these signs were low and did not show a relationship to treatment.

Occurrences of wounds (open or with crusts) or hairloss/erythema/discoloration on the leg, paw, snout, cervical region or ear and localised swelling/thickening on the cheek, cervical, leg or abdominal region recorded in individual animals in all groups did not show a relationship to dose and were considered not to be related to treatment with the test item.

The transient lameness in one control animal occurred during animal interaction in the morning exercise period. Additionally, signs of gingivitis in one male and one female and conjunctivitis in one female of the high dose group were observed but were considered not to be test item related due to the low incidence.

The females were examined regularly during the study from commencement of pretest for signs of oestrus, evidence of which was observed in all animals except one 125 mg/kg/day female during the study. This observation is consistent with the age of the dogs.

Veterinary examination: There were no findings recorded in the veterinary examination which were considered to be related to treatment with the test item. The findings seen in the pretest or in single animals were considered not to be treatment related.

Bodyweight: Mean body weight and body weight gain were unaffected throughout the treatment period in all dose groups. Absolute body weights are reported in table 2.

Table 2: Mean intergroup comparison of absolute bodyweights (kg) by week

	Dietary Concentration of bicyclopyrone (mg/kg/day)							
	Males				Females			
Week*	**0**	**2.5**	**25**	**125**	**0**	**2.5**	**25**	**125**
Pre-Test Week 2	8.4 ± 0.8	8.6 ± 0.7	8.8 ± 0.9	8.3 ± 1.0	7.7 ± 0.7	7.7 ± 0.7	7.4 ± 0.4	7.6 ± 0.6
Week 1	8.6 ± 0.7	8.8 ± 0.8	9.2 ± 0.9	8.7 ± 1.0	7.7 ± 0.6	7.9 ± 0.7	7.7 ± 0.4	7.8 ± 0.6
Week 14	9.8 ± 1.3	9.6 ± 0.5	10.2 ± 1.1	10.3 ± 1.0	8.4 ± 0.8	8.9 ± 0.8	8.6 ± 0.3	8.6 ± 1.1
Week 27	10.6 ± 1.3	9.8 ± 0.5	10.9 ± 1.5	10.8 ± 1.6	9.0 ± 0.9	8.9 ± 0.9	8.3 ± 0.4	8.8 ± 1.2
Week 52	11.5 ± 2.0	10.2 ± 0.6	11.3 ± 2.1	10.3 ± 0.5	9.5 ± 1.0	9.3 ± 1.1	8.7 ± 0.3	9.2 ± 1.2

Data were taken from pages 67-78 of the study report
*In the study report, the durations for this table are reported as days as opposed to weeks

Food consumption: Intermittently lower food intake was noted in individual females (particularly number 30) dosed with 125 mg/kg bicyclopyrone. Group mean food consumption was not affected throughout the treatment period in either sex.

Ophthalmoscopic examination: Persistent corneal opacities which developed over the course of treatment were observed in two males and one female at 25 mg/kg/day and in one female at 125 mg/kg/day. One male (no. 12) dosed at 25 mg/kg/day and one female (no. 29) dosed at 125 mg/kg/day showed sensitivity to light during the examination.

The corneal opacity noted pre-test in high dose male no. 14 was initially typical of those lesions seen in untreated animals and changed into the characteristic treatment related star shaped opacity during the course of the study. This finding is concluded to be treatment related.

There were no ophthalmoscopic changes which were considered to be related to treatment with the test item in animals dosed at 2.5 mg/kg/day.

Transient corneal opacities were noted in two females (nos. 22 and 23) dosed at 2.5mg/kg/day at week 26 only. The transient nature of these findings and the fact that similar opacities were noted in two control females (nos. 18 and 19) led to the conclusion that the findings at 2.5 mg/kg were not treatment-related.

There were no other ophthalmoscopic changes which were considered to be related to treatment with the test item. The remaining findings were typical of observations commonly seen spontaneously in dogs at this laboratory.

Haematology: There were no differences in haematology parameters considered to be related to treatment with the test item.

Some intergroup variations occasionally achieved statistical significance, but these did not show a relationship to dose level or reflected differences which were present during pretest and were considered to reflect normal biological variation.

Clinical chemistry: Decreased plasma cholesterol levels were recorded in males and females dosed at 125 mg/kg/day in weeks 13, 26 and 52 (↓41-57%) and in females at week 13 only when treated with 25 mg/kg/day (↓26%). The mean values were generally statistically significant for both sexes. See table 3.

Statistically significantly lower glucose levels were recorded in females dosed at 125 mg/kg/day in week 52 when compared with concurrent control values (↓18%). The values are within the range of laboratory historical control values and are therefore considered unrelated to treatment. See table 3.

Statistically significantly lower total bilirubin levels were noted in females dosed with 25 and 125 mg/kg/day in week 52 (↓42% and ↓44%). The values are within the range of laboratory historical control values and are therefore considered unrelated to treatment. See table 3.

Some intergroup variations occasionally achieved statistical significance, but these did not show a relationship to dose level or reflected differences which were present during pretest or in the controls and were considered to reflect normal biological variation.

Table 3: Intergroup comparison of clinical chemistry results

	Dose Level of bicyclopyrone (mg/kg/day)							
	Males				Females			
Parameter	0	2.5	25	125	0	2.5	25	125
Glucose (mmol/L)								
Pre-test	5.76 ±0.38	5.86 ±0.37	5.69 ±0.26	5.27 ±0.64	5.82 ±0.18	5.80 ±0.24	5.41 ±0.21	5.41 ±0.58
Week 13	5.69 ±0.25	5.91 ±0.32	5.95 ±0.57	5.73 ±0.39	6.08 ±0.45	6.27 ±0.46	5.71 ±0.31	5.60 ±0.63
Week 26	5.96 ±0.12	5.78 ±0.20	5.80 ±0.33	5.66 ±0.56	5.99 ±0.60	5.78 ±0.26	5.59 ±0.54	5.15 ±0.51
Week 52	5.16 ±0.25	5.32 ±0.12	5.47 ±0.37	4.76 ±0.23	5.45 ±0.60	5.57 ±0.47	5.30 ±0.41	4.49* ±0.19 (↓18%)
Bilirubin (µmol/L)								
Pre-test	1.42 ±0.16	1.66 ±0.58	1.29 ±0.24	1.19 ±0.17	1.67 ±0.40	1.60 ±0.29	1.82 ±0.38	1.84 ±0.46
Week 13	1.97 ±0.55	1.64 ±0.34	1.50 ±0.47	1.82 ±0.35	2.48 ±0.75	1.62 ±0.34	2.21 ±0.52	2.07 ±0.30
Week 26	1.69 ±0.30	1.50 ±0.41	1.22 ±0.21	1.66 ±0.15	2.26 ±0.41	2.00 ±0.37	2.15 ±0.53	2.05 ±0.65
Week 52	2.18 ±0.15	2.00 ±0.34	1.75 ±0.13	2.10 ±0.35	3.25 ±0.94	2.65 ±0.75	1.88* ±0.28 (↓42%)	1.83* ±0.13 (↓44%)
Cholesterol (mmol/L)								
Pre-test	3.36 ±0.43	3.42 ±0.54	3.13 ±0.49	3.11 ±0.24	3.21 ±0.37	3.20 ±0.13	2.90 ±0.44	3.39 ±0.54
Week 13	3.83 ±0.52	3.77 ±0.69	3.17 ±0.69	1.82 ±0.30	3.89 ±0.37	3.95 ±0.60	2.87* ±0.28 (↓26%)	1.68** ±0.45 (↓57%)
Week 26	3.06 ±0.42	3.23 ±0.36	2.61 ±0.76	1.48** ±0.35 (↓52%)	3.88 ±1.36	3.53 ±0.34	2.61 ±0.38	1.84** ±0.61 (↓53%)
Week 52	3.30 ±0.60	3.06 ±0.48	2.55 ±0.62	1.54** ±0.10 (↓53%)	4.10 ±1.19	3.90 ±0.31	3.15 ±0.60	1.83** ±0.46 (↓56%)

Data taken from pages 146-161 of the study report
*Significant at 5%
**Significant at 1%

Urinalysis: There were no changes in urinalysis parameters considered to be related to treatment with the test item.

Ketones were apparently detected in the urine of all animals after treatment with bicyclopyrone during week 1, 6 and 13. Further laboratory investigations demonstrated that the tyrosine derived metabolite HPPA can cause a red-brown change in the urine dipstick used to measure ketones. When using an automated method, this colour change was incorrectly identified as the presence of ketones in the urine.

Visual examination can clearly distinguish between the red-brown change caused by HPPA and the purple-violet change produced by acetoacetate. As an HPPD inhibitor, bicyclopyrone blocks the conversion of HPPA to homogentisate following HPPA production from tyrosine leading to elevated HPPA levels. The apparent detection of ketones in bicyclopyrone-treated animals in this study was due to cross-reactivity of HPPA with the dip-stick used to measure ketones.

Toxicokinetic Evaluation of bicyclopyrone, CSAA915194 and Tyrosine Levels:
Toxicokinetic data are summarized in tables 4, 5 and 6. Several samples from the control group gave quantifiable concentrations of the parent compound of bicyclopyrone (NOA449280; 103 to 328 ng/mL) and/or metabolite (CSAA915194; 1.0 to 32.1 ng/mL). Although, these concentrations were above the limit of quantification (100 ng/mL and 1.0 ng/mL for bicyclopyrone and metabolite, respectively) of the assay they were low when compared to the treated low dose animals. The reason for quantifiable concentrations being obtained in the control samples is unknown but at such concentrations, the study was not considered to be compromised. The reference item tyrosine is an endogenous compound and therefore, as expected, quantitative concentrations of tyrosine in the control male and female dogs were found.

Exposure was primarily to the parent compound. The metabolite reached only 0.8 to 5.4 % of the AUCτ of bicyclopyrone. In general, bicyclopyrone reached peak plasma concentration at 2h (t_{max}) post-dosing.

With a 50-fold dose range investigated, exposure to bicyclopyrone increased with AUCτ ratios of 15 to 42. For CSAA915194, AUCτ increased in males with ratios of 23 to 52 and in females with ratios of 41 to 55.

Following treatment with bicyclopyrone at 2.5 mg/kg/day, tyrosine AUCτ increased by a factor of 6.2 to 22 when compared to controls. No further increase in AUCτ of tyrosine (ratios: 0.9 to 1.3) was found on increasing bicyclopyrone doses to 25 and 125 mg/kg/day.

No consistent gender difference was found throughout all treated groups and at all occasions for parent, metabolite and tyrosine; except at 2.5 mg/kg/day, where exposure to CSAA915194 was lower in females than in males.

At each bicyclopyrone treatment level, exposure to parent, its metabolite and tyrosine remained similar after 4, 13, 26 and 52 weeks of repeated dosing.

Table 4: Intergroup comparison of plasma bicyclopyrone toxicokinetic parameters

Males	Group	2*	3	4
Dose	[mg/kg/day]	2.5	25	125
		Week 4		
C_{max}	[ng/mL]	5101 ± 1074	30613 ± 7036	151531 ± 34243
t_{max} (range)	[h]	1-8	2-8	2
AUC_{τ}	[ng•h/mL]	81788 ± 14869	394468 ± 43002	1303532 ± 278123
		Week 13		
C_{max}	[ng/mL]	8870 ± 1944	36111 ± 9524	147088 ± 53074
t_{max} (range)	[h]	1-2	2-8	1-2
AUC_{τ}	[ng•h/mL]	92053 ± 17132	352235 ± 47257	1344782 ± 499453
		Week 26		
C_{max}	[ng/mL]	7022 ± 1744	46560 ± 15497	224333 ± 38354
t_{max} (range)	[h]	1-2	1-3	2-3
AUC_{τ}	[ng•h/mL]	59091 ± 20061	406764 ± 40336	2461818 ± 969692
		Week 52		
C_{max}	[ng/mL]	7871 ± 3747	29459 ± 6960	218324 ± 108961
t_{max} (range)	[h]	0-2	2-8	2-4
AUC_{τ}	[ng•h/mL]	80453 ± 22179	503148 ± 141229	2107091 ± 1076475

*Dog 5 excluded from mean calculation

Females	Group	2	3	4
Dose	[mg/kg/day]	2.5	25	125
		Week 4		
C_{max}	[ng/mL]	5822 ± 1073	41748 ± 4538	172839 ± 42141
t_{max} (range)	[h]	2-8	1-2	2-3
AUC_{τ}	[ng•h/mL]	106604 ± 26746	463466 ± 171660	1684944 ± 642158
		Week 13		
C_{max}	[ng/mL]	9235 ± 1007	42730 ± 6184	141030 ± 19070
t_{max} (range)	[h]	1	1	2-3
AUC_{τ}	[ng•h/mL]	71674 ± 23555	393731 ± 132993	1260425 ± 582045
		Week 26		
C_{max}	[ng/mL]	7862 ± 2115	60089 ± 6656	184304 ± 45540
t_{max} (range)	[h]	1	1-2	2-3
AUC_{τ}	[ng•h/mL]	75160 ± 13864	508004 ± 176360	1307388 ± 221681
		Week 52		
C_{max}	[ng/mL]	7300 ± 2859	46888 ± 4107	184197 ± 34009
t_{max} (range)	[h]	1-2	1-2	2
AUC_{τ}	[ng•h/mL]	72892 ± 28145	501996 ± 215769	1517190 ± 556161

Data were taken from page 420 of the study report

Table 5: Intergroup comparison of plasma CSAA915194 toxicokinetic parameters

Males	Group	2*	3	4
Dose	[mg/kg/day]	2.5	25	125
		Week 4		
C_{max}	[ng/mL]	85.2 ± 30.0	668 ± 165	3171 ± 817
t_{max} (range)	[h]	1-12	3-12	3-4
AUC_{τ}	[ng•h/mL]	1219 ± 283	10448 ± 1427	36262 ± 5299
		Week 13		
C_{max}	[ng/mL]	172 ± 48	727 ± 192	2538 ± 616
t_{max} (range)	[h]	0-24	2-8	2-4
AUC_{τ}	[ng•h/mL]	1505 ± 204	9038 ± 3496	34215 ± 8558
		Week 26		
C_{max}	[ng/mL]	93.5 ± 35.9	688 ± 156	2937 ± 487
t_{max} (range)	[h]	1-2	0-3	3-8
AUC_{τ}	[ng•h/mL]	802 ± 312	8014 ± 2560	41948 ± 11628
		Week 52		
C_{max}	[ng/mL]	193 ± 156	596 ± 437	2715 ± 840
t_{max} (range)	[h]	0-2	3-12	3-4
AUC_{τ}	[ng•h/mL]	1066 ± 586	11213 ± 8376	35757 ± 10742

*Dog 5 excluded from mean calculation

Females	Group	2	3	4
Dose	[mg/kg/day]	2.5	25	125
		Week 4		
C_{max}	[ng/mL]	48.5 ± 16.9	750 ± 167	2937 ± 322
t_{max} (range)	[h]	0-12	2-12	3
AUC_{τ}	[ng•h/mL]	914 ± 474	11437 ± 5227	43930 ± 4638
		Week 13		
C_{max}	[ng/mL]	64.9 ± 21.8	712 ± 109	2562 ± 334
t_{max} (range)	[h]	1-2	2-3	3-4
AUC_{τ}	[ng•h/mL]	585 ± 311	8473 ± 2521	32062 ± 9325
		Week 26		
C_{max}	[ng/mL]	67.3 ± 41.7	672 ± 54	2427 ± 864
t_{max} (range)	[h]	2	2-3	2-3
AUC_{τ}	[ng•h/mL]	629 ± 272	8424 ± 2969	25910 ± 8052
		Week 52		
C_{max}	[ng/mL]	90.1 ± 91.2	625 ± 139	2654 ± 603
t_{max} (range)	[h]	0-2	2-3	3
AUC_{τ}	[ng•h/mL]	707 ± 507	9097 ± 3729	32002 ± 8277

Table 6: Intergroup comparison of plasma tyrosine toxicokinetic parameters

Males	Group	1	2	3	4
Dose	[mg/kg/day]	0	2.5	25	125
			Week 4		
C_{max}	[µg/mL]	39.8 ± 26.6	299 ± 40	350 ± 18	349 ± 29
t_{max} (range)	[h]	3	4-12	8	8
AUC_{τ}	[µg•h/mL]	524 ± 301	6002 ± 1088	7351 ± 257	7286 ± 722
			Week 13		
C_{max}	[µg/mL]	39.5 ± 15.4	358 ± 55	363 ± 36	377 ± 75
t_{max} (range)	[h]	3-12	3-4	4-12	4-8
AUC_{τ}	[µg•h/mL]	537 ± 88	7089 ± 1527	7696 ± 683	7626 ± 1127
			Week 26		
C_{max}	[µg/mL]	74.6 ± 40.9	329 ± 65	322 ± 12	338 ± 39
t_{max} (range)	[h]	4	3-4	4-8	3-8
AUC_{τ}	[µg•h/mL]	1072 ± 482	6608 ± 1403	6613 ± 433	7272 ± 748
			Week 52		
C_{max}	[µg/mL]	62.8 ± 11.0	318 ± 52	323 ± 20	347 ± 36
t_{max} (range)	[h]	2-3	4	4-12	4-8
AUC_{τ}	[µg•h/mL]	717 ± 183	6444 ± 1186	6956 ± 587	7476 ± 903

Females	Group	1	2	3	4
Dose	[mg/kg/day]	0	2.5	25	125
			Week 4		
C_{max}	[µg/mL]	23.0 ± 5.4	317 ± 28	354 ± 42	370 ± 38
t_{max} (range)	[h]	2	4-8	2-12	4-8
AUC_{τ}	[µg•h/mL]	309 ± 134	6753 ± 584	7121 ± 1005	7676 ± 1032
			Week 13		
C_{max}	[µg/mL]	45.7 ± 7.7	415 ± 96	399 ± 55	341 ± 61
t_{max} (range)	[h]	2-4	1-8	3-12	4-12
AUC_{τ}	[µg•h/mL]	542 ± 41	7760 ± 291	8232 ± 1380	7035 ± 951
			Week 26		
C_{max}	[µg/mL]	42.0 ± 5.3	306 ± 29	362 ± 40	411 ± 53
t_{max} (range)	[h]	3	3-8	4	4-8
AUC_{τ}	[µg•h/mL]	543 ± 100	6405 ± 558	7349 ± 936	8676 ± 844
			Week 52		
C_{max}	[µg/mL]	47.5 ± 21.3	298 ± 40	356 ± 41	395 ± 63
t_{max} (range)	[h]	2-4	3-4	3-4	3-12
AUC_{τ}	[µg•h/mL]	516 ± 127	6415 ± 891	7052 ± 1091	8131 ± 1088

Sacrifice and pathology:

Organ weights: There were no organ weight changes recorded which were considered to be related to treatment with the test item. Absolute liver weights are listed in table 7.

Table 7: Absolute Liver Weights (g)

Dose Level of bicyclopyrone (mg/kg/day)							
Males				Females			
0	2.5	25	125	0	2.5	25	125
329.6 ± 64.8	354.1 ± 27.3	355.8 ± 78.2	367.5 ± 67.5	324.4 ± 87	296.3 ± 36.5	288.3 ± 39.2	320 ± 43

Data were taken from pages 171 and 177 of the study report

Macroscopic findings: There were no macroscopic findings recorded which were considered to be related to treatment with the test item.

All findings were considered to be incidental and commonly occur in dogs of this strain and age under the experimental conditions used in this study.

Microscopic findings: Microscopic examination of the tissues from the male (no. 15) treated at 125 mg/kg/day and found dead in week 48 did not reveal any changes explaining the death. The relevance of this mortality is unclear.

Microscopic findings considered to be related to treatment with the test item were seen in the nervous system in animals of all treated groups.

Minimal to moderate chromatolysis and swelling of selected neurons occurred in the dorsal root ganglia in two males and three females at 2.5 mg/kg/day (50% and 75%), all males and females at 25 mg/kg/day (100%), and three males and two females at 125 mg/kg/day (75% and 50%). There was no clear dose-effect relationship with respect to the incidence and mean grade of this finding. However, as it occurred in animals treated with the test item only and is not known to occur as a background finding, it is considered to represent an effect of the test item. See table 5.

Minimal degeneration of nerve fibers of the sciatic nerve was observed in one control male (25%), two males (50%) and one female (25%) at 2.5 mg/kg/day, two males (50%) and one female (25%) at 25 mg/kg/day and two males and two females at 125 mg/kg/day (both 50%). See table 5.

Minimal to slight degeneration of nerve fibers occurred in the spinal nerve roots of one control male, two males at 2.5 mg/kg/day, and one male and one female at 125 mg/kg/day showing no dose response for this effect.

There were no other microscopic findings considered to be related to treatment with the test item.

All other findings were considered to be incidental and within the range of background alterations recorded in dogs of this strain and age. Neither the incidence nor distribution or morphologic characteristics indicated a relationship to treatment with the test item.

Table 5: Incidence and Grading of Chromatolysis within the Doral Root Ganglia of Dogs

Histopathology	Males Dose level (mg/kg)				Females Dose level (mg/kg)			
	0	**2.5**	**25**	**125**	**0**	**2.5**	**25**	**125**
Number of animals examined	4	4	4	4	4	4	4	4
Ganglion, dorsal root exam.	4	4	4	4	4	4	4	4
Chromatolysis/ swelling								
Grade 1	-	1	1	1	-	1	2	-
Grade 2	-	-	2	2	-	2	1	2
Grade 3	-	1	1	-	-	-	1	-
Total affected	-	2 (50%)	4 (100%)	3 (75%)	-	3 (75%)	4 (100%)	2 (50%)
Mean grade/tissue affected	-	2.0	2.0	1.7	-	1.7	1.8	2.0
Sciatic Nerve, exam.	4	4	4	4	4	4	4	4
Degeneration								
Grade 1	1	2	2	2	-	1	1	2
Grade 2	-	-	-	-	-	-	-	-
Grade 3	-	-	-	-	-	-	-	-
Total affected	1 (25%)	2 (50%)	2 (50%)	2 (50%)	-	1 (25%)	1 (25%)	2 (50%)
Mean grade/tissue	1.0	1.0	1.0	1.0	-	1.0	1.0	1.0

Data were taken from pages 652, 660, 668, 676 of the study report.

INVESTIGATOR'S CONCLUSIONS: Oral administration of bicyclopyrone for 52 weeks at dose levels of 2.5, 25 and 125 mg/kg/day resulted in persistent opacity of the cornea in the eyes of some dogs dosed with 25 and 125 mg/kg/day.

Minimal microscopic changes which resulted in peripheral neuropathy were noted in the nervous system of dogs in all dose groups. Based on the results of this study, the no observed adverse effect level (NOAEL) was considered to be below 2.5 mg/kg/day.

REVIEWER'S COMMENTS:

Based on the results of this study, the LOAEL is 2.5 mg/kg/day based upon an increased incidence of chromatolysis and swelling of selected neurons in the dorsal root ganglia and degeneration of nerve fibers in the sciatic nerve roots in both sexes at all treatment levels. A NOAEL was not observed.

EPA and PMRA (Canada) agree on the NOAEL/LOAEL statement for this study. APVMA/OCS (Australia) feels that the NOAEL should be 2.5 mg/kg/day and the LOAEL should be 25 mg/kg/day based upon minor eye effects. APVmA is further of the opinion that the histophathological effects in the nervous system are not adverse based upon the supplied discussion paper (Botham and Wright, 2012; Project ID TK0144119).

This study is classified as totally reliable (**acceptable/guideline)** and satisfies the guideline requirements for a chronic oral study [OPPTS 870.4100, OECD 452] in dogs. EPA and PMRA

(Canada), and APVMA/OCS (Australia) agree on the classification but not the regulatory decision for this study.

(Braun, L, 2010)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anw J. Dnbr
Risk Assessment Branch I, Health Effects Division (7509P) Date: 03/18/15
EPA Reviewer: Greg Akerman, Ph.D. **Signature:** Greg Ak
Risk Assessment Branch I, Health Effects Division (7509P) Date: 3/18/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Carcinogenicity – Mouse (feeding)
OECD 451 (2009): OPPTS 870.4200 (1998): 88/302/EEC B.32 (2001): JMAFF 12 Nohsan No. 8147 (2000)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5% w/w)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[[2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]- ; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one; bicyclopyrone, SYN449280

CITATION: Robertson B, 2012. NOA449280: 80 week mouse dietary carcinogenicity study. Charles River, Tranent, Edinburgh, EH33 2NE, UK. Laboratory Report No. 30195, 17 August 2012. Unpublished. (Syngenta File No. NOA449280_11243). MRID 47841987

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a carcinogenicity study in mice (MRID #47841987), bicyclopyrone (NOA449280, purity 94.5% w/w) was administered to groups of 50 male and 50 female CD-1 mice in the diet at dose levels of 0, 70, 1700 and 7000 ppm (equivalent to 0, 8.7 / 9.2, 233 / 242, 940 / 1027 mg/kg bw/day for males / females respectively) for a period of at least 80 weeks.

Animals were monitored regularly for viability and for signs of ill health or reaction to treatment. Body weights and food consumption were measured and recorded at predetermined intervals from pretrial up until the completion of treatment. At week 80, prior to terminal kill, blood samples were collected from all surviving animals for haematological analysis. Blood films were made from all surviving animals during week 53/54 and at week 80; however, blood cell morphology was not performed as no treatment related effects were seen on white cell haematological parameters at termination. All surviving animals were terminated and subjected to a detailed necropsy examination after completion of treatment. Tissues from all animals were subject to a comprehensive histological evaluation.

There were no treatment related clinical observations and no effects on mortality at any dose.

At all doses, there were changes at 70 and 1700 ppm, such as increased absolute and adjusted liver weights which were considered adaptive.

At 7000 ppm bicyclopyrone, there was a statistically significant decrease in the absolute body weights in females (↓9-12%).

At the highest dose tested, there was an increased incidence of lung adenomas which were determined to not be treatment related and thus not toxicologically significant.

Based upon the effects in this study, the LOAEL is 7000 ppm (1027 mg/kg/day [F]) based upon decreased absolute body weights in females. The NOAEL is 1700 ppm (242 mg/kg/day [F]). The NOAEL for males is 7000 ppm (940 mg/kg/day). The LOAEL for males was not established.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirements (OPPTS 870.4200) for an oncogenicity study in the mice.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided. Deviations occurring during the study were minor and did not compromise the quality of the study.

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Technical material, brown/beige powder
Lot/Batch number:	SEZ3AP006/MILLED
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Reanalysis March 2011

Structure:

Vehicle and/or positive control: The test substance was administered via Rat and Mouse (modified) No. 1 Diet SQC Expanded (Ground) (Special Diets Services Limited, 1 Stepfield, Witham, Essex, UK).

Test Animals:

Species	Mouse
Strain	CD-1 mice (Crl:CD-1(ICR))
Age/weight at dosing	Approximately 6 weeks / 26.1-40.9 g (males), 22.6-32.2 g (females)
Source	Charles River UK Limited, Margate, Kent, UK
Housing	Male animals housed individually and females up to 3 per cage in suspended polycarbonate cages with stainless steel grid tops.
Acclimatisation period	Approximately 2 weeks
Diet	Rat and Mouse (modified) No. 1 Diet SQC Expanded (Ground) (Special Diets Services Limited, 1 Stepfield, Witham, Essex, UK) *ad libitum*
Water	Mains water *ad libitum*
Environmental conditions	Temperature: 12-26°C (intended range 19-23°C) Humidity: 29.58-80.33% (intended range 40-70%) Air changes: Minimum of 15/hour Photoperiod: 12 hours light / 12 hours dark

In-life dates: Start: 04 October 2007 End: 09 September 2009

Study Design and Methods: In a carcinogenicity study NOA449280 (purity 94.5% w/w) was administered to groups of 50 male and 50 female CD-1 mice in the diet at dose levels of 0, 70, 1700 and 7000 ppm (equivalent to 0 / 0, 8.7 / 9.2, 233 / 242, 940 / 1027 mg/kg bw/day for males / females respectively) for a period of at least 80 weeks.

Animal assignment: On arrival from the suppliers, the animals were allocated to cages on racks. Cages were racked by treatment group and vertically throughout the rack. Each month from the commencement of pretrial, each column of cages on a rack were moved one position along the racks assigned to that sex, with the end column returning to the start of the first rack, to minimise environmental effects. The control animals were housed on a separate rack from the treatment groups. During pretrial, group mean body weights were checked to ensure that all groups had a similar body weight for each sex.

Table 1: Study design

Test group	Dietary concentration (ppm)	# male	# female
Control	0	1-50	201-250
Low	70	51-100	251-300
Mid	1700	101-150	301-350
High	7000	151-200	351-400

Table was taken from pages 21 of the study report

Diet preparation and analysis: Experimental diets were prepared by direct admixture of test item to a required amount of untreated diet and blended for 20 minutes in a diet mixer. Blank diet (without the test substance under investigation) was prepared for Control animals. Diet formulations were prepared and dispensed once every 2 weeks.

Prior to study commencement, stability data were generated by Charles River, Edinburgh for 15 days for experimental diets stored at -20ºC in the dark, in the concentration range of 2.5-7000 ppm. During the study, triplicate samples (3 x 50 g) were taken from each experimental diet (including control) at approximately 3 monthly intervals, immediately after preparation, and analysed for achieved concentration and homogeneity.

Concentration analysis results:
Analysed concentrations of test item in experimental diets were found to be within ±4.7% (Week 1), ±2.1% (Week 13), ±3.9% (Week 26), ±1.3% (Week 39), ±0.7% (Week 52), ±3.2% (Week 65) and ±0.6% (Week 79) of the theoretical concentrations.

Homogeneity results:
The coefficient of variance was low (5.5% or below) indicating satisfactory homogeneity of diet formulations.

Stability results:
Stability for the range 2.5-7000 ppm was satisfactory for 15 days when stored at ambient temperature and protected from light, or when frozen at -20°C.

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable, provided that the cited stability study did indicate that the test compound was stable under conditions of the study.

Observations: All animals were checked early morning and as late as possible each day for signs of viability. Once each week all animals received a detailed clinical examination, including appearance, movement and behaviour patterns, skin and hair condition, eyes and mucous membranes, respiration and excreta.

Body weight: Body weights were recorded once weekly during pretrial up until week 14 of treatment, and approximately once every 2 weeks from week 15 up until the end of treatment.

Food consumption, utilization and test substance intake: The quantity of food consumed by each cage of animals was measured and recorded once weekly during pretrial up until week 14 of treatment and once every 4 weeks from week 16 up until the end of treatment.

Food utilization was calculated for weeks 1-4, 5-8, 9-13 and 1-13 according to the following formula:

(Cage mean weight gain x 100) / cage total food consumption

The amount of test item ingested was calculated at regular intervals during treatment using the following formula:

$$\text{Achieved intake (mg/kg/day)} = \frac{\text{Nominal Concentration (ppm)} \times \text{Food Consumption (g/day)}}{\text{Mid-point Body Weight (g)}}$$

Water consumption: Water consumption was qualitatively monitored by visual inspection of water bottles on a weekly basis throughout the study.

Haematology: Blood was collected from all surviving animals via the orbital sinus under isoflurane anaesthesia and transferred into tubes containing EDTA prior to the terminal kill. The animals were not deprived of food overnight prior to sampling. The following parameters were examined:

total white cell count differential white cell count

A blood film smear was made from all EDTA haematology samples and stained for possible examination from all surviving animals at week 53/54 and week 80 scheduled euthanasia and

femoral bone marrow smears were taken at necropsy and stored for possible evaluation. Neither the blood nor bone marrow smears were examined as haematological findings indicated that evaluation would not yield any further information.

Investigations *post mortem:* After at least 80 weeks of treatment all surviving animals were killed in random order by exposure to carbon dioxide and had their terminal body weight recorded followed by exsanguination.

Macroscopic examination: All animals were examined *post mortem*. The necropsy consisted of a complete internal and external examination which included body orifices (ears, nostrils, mouth, anus, vulva) and cranial, thoracic and abdominal organs and tissues.

Organ weights: From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

adrenal glands
brain
epididymides
heart
kidneys
liver and gall bladder
ovaries
spleen
testes
uterus (with cervix)

Paired organs were weighed separately and the sum of the individual organs used for reporting purposes.

Tissue submission / microscopic examination: The following tissues were examined *in situ*, removed and examined and fixed in an appropriate fixative, and 4-6 µm sections all processed tissues were examined by light microscopy:

abnormal tissue (including local lymph nodes to masses)
adrenal gland
aortic arch
tongue
brain (forebrain, midbrain, cerebellum and pons)
caecum
colon
duodenum
epididymis
eyes
femur (including knee joint and bone marrow)
Harderian gland
heart
lachrymal gland
ileum
jejunum
kidney
larynx
liver and gall bladder
lung
optic nerve
oesophagus
ovary
oviduct
vagina
peyer's patches
pancreas
parathyroid gland
pharynx
pituitary gland
prostate gland
rectum
salivary gland
seminal vesicle
spinal cord (cervical, midthoracic, lumbar)
skin
spleen
sternum (including bone marrow)
stomach
testis
thymus
thyroid gland

lymph node - mesenteric	trachea
mammary gland	urinary bladder
nerve - sciatic	uterus
nasal cavity	thigh muscle

Statistics: Body weight, cumulative body weight gain, food consumption, food utilization, haematology and organ weight data were analysed using a parametric ANOVA and pairwise comparisons made using the Dunnett's t-test. Organ weights were also analysed by analysis of covariance (ANCOVA) using terminal kill body weight as covariate. Analyses of variance and covariance were carried out using the MIXED procedure in SAS (9.1.3). Differences from control were tested statistically by comparing each treatment group least-squares mean with the control group least-squares mean using a two-sided Dunnett's t-test, based on the error mean square in the analysis. All statistical tests were two sided and performed at the 5% and 1% levels.

The Dunnett's test was performed for all continuous data parameters, regardless of whether the initial ANOVA or ANCOVA was statistically significant.

Kaplan-Meier survival estimates were calculated separately for each sex and treatment group. Pairwise comparisons of the incidence of tumor and histological lesions was made using Fisher's Exact test (two-tailed). Histological findings with multiple severities were also analysed using the Mann-Whitney U test. Further analyses were performed using Peto's time adjusted methods.

The statistical evaluation of the tumor data was performed in SAS (v8.2) using PROC MULTTEST. Methods used for the age-adjusted analysis of fatal and non-fatal tumors were based on the IARC guidelines.

RESULTS AND DISCUSSION

Mortality: There were no statistically significant differences in mortality for either male or female treated groups in comparison to their respective controls. Additionally, the trend test was not seen to be statistically significant for any male or female treated group.

Clinical observations: There were a variety of clinical observations recorded in control and treated mice but these were either commonly seen observations in this age and strain of mouse or were seen in small numbers of animals. None of the observations seen were considered to be related to treatment./

Bodyweight and weight gain: Absolute body weight and body weight gain data are presented in tables 2 and 3. Males and females treated at 7000 ppm showed slight decreases in absolute body weight (↓8-9% and ↓9-12%, respectively) and body weight change (17-29 and 23-55%, respectively) when compared to their control, although males treated at 7000 ppm had a statistically significantly lower initial body weight. Statistically significant differences were noted at various timepoints and the slight decreases were consistent throughout the treatment period.

Males treated at 1700 ppm showed a statistically significantly lower (↓4%) initial body weight compared to their control. However, no consistent statistically significant difference was noted after the first few weeks of the study.

Females treated at 1700 and 7000 ppm showed isolated statistically significantly higher body weight gains Weeks 0-2 (↑40% and ↑50%), and females treated at 70 ppm were noted to have an isolated statistically significantly lower body weight change during Weeks 0-3. These isolated differences were not related to treatment.

Table 2: Mean intergroup comparison of absolute bodyweights (g) by week

	Dietary Concentration of bicyclopyrone (ppm)							
	Males				Females			
Week*	**0**	**70**	**1700**	**7000**	**0**	**70**	**1700**	**7000**
Pre-Test (Week 0)	35.3 ± 2.4	35.0 ± 2.8	33.9* ± 2.9 (↓4%)	33.9* ± 2.7 (↓4%)	26.9 ± 1.8	27.0 ± 1.6	26.9 ± 1.8	26.5 ± 2.0
Week 2	38.2 ± 2.8	38.3 ± 3.3	37.5 ± 3.6	37.2 ± 3.0	28.8 ± 2.3	28.6 ± 2.1	29.6 ± 2.3	29.5 ± 2.4
Week 20	54.7 ± 6.1	54.2 ± 6.4	51.2* ± 6.8 (↓6%)	49.9** ± 5.9 (↓9%)	42.8 ± 6.2	42.9 ± 8.2	43.7 ± 8.0	39.0* ± 5.3 (↓9%)
Week 40	58.7 ± 7.0	59.1 ± 7.2	56.2 ± 7.6	54.2** ± 6.8 (↓8%)	49.9 ± 8.1	52.0 ± 10.9	50.2 ± 10.2	44.1** ± 7.0 (↓12%)
Week 80	61.9 ± 7.7	62.8 ± 8.1	58.4 ± 7.5	57.0* ± 8.4 (↓8%)	56.0 ± 8.7	58.5 ± 12.6	54.2 ± 13.0	51.4 ± 8.8

Data were taken from pages 74-81 of the study report

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Table 3: Intergroup comparison of bodyweight gain (g) - selected timepoints

	Dietary Concentration of bicyclopyrone (ppm)							
	Males				Females			
weeks	**0**	**70**	**1700**	**7000**	**0**	**70**	**1700**	**7000**
0-1	2.1 ± 0.7	2.3 ± 0.8	1.5* ± 0.8 (↓29%)	1.5* ± 0.8 (↓29%)	1.2 ± 1.0	0.6 ± 1.1	1.4 ± 1.5	1.1 ± 1.4
0-2	2.9 ± 1.2	3.2 ± 1.2	3.6** ± 1.5 (↑24%)	3.4 ± 1.1	2.0 ± 1.4	1.6 ± 1.5	2.8* ± 1.5 (↑40%)	3.1** ± 1.4 (↑55%)
0-4	5.9 ± 1.9	6.4 ± 2.1	5.9 ± 2.1	4.9* ± 1.7 (↓17%)	4.1 ± 1.9	4.2 ± 1.8	4.3 ± 1.9	3.8 ± 2.0
0-8	10.7 ± 3.1	10.9 ± 3.4	10.1 ± 3.5	8.7** ± 2.4 (↓19%)	7.7 ± 3.1	8.2 ± 3.8	7.6 ± 3.5	7.2 ± 3.4
0-26	22.5 ± 5.7	22.2 ± 5.5	19.8 ± 6.4	17.9** ± 5.3 (↓20%)	19.6 ± 6.6	21.5 ± 8.6	18.8 ± 8.3	14.0** ± 5.0 (↓29%)
0-52	26.4 ± 6.9	27.0 ± 6.7	25.3 ± 7.6	23.0* ± 6.5 (↓13%)	27.9 ± 8.5	29.7 ± 10.5	27.3 ± 10.4	21.6** ± 7.7 (↓23%)
0-80	26.5 ± 7.2	27.9 ± 6.9	24.7 ± 7.1	23.3 ± 7.7	29.4 ± 8.2	31.4 ± 12.3	27.3 ± 12.3	24.9 ± 8.4

Data were taken from pages 82-89 of the study report

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Food consumption, utilization and compound intake: Males treated at 1700 and 7000 ppm had statistically significantly lower food consumption than controls during pretrial, thereafter

there was no consistent pattern and food consumption values were both lower and higher than control values. There was no effect on food consumption in males at 70 ppm or females at any dose level.

Food utilization data are presented in table 4. Food was less efficient in males treated at 7000 ppm from Weeks 1-4 and 5-8 and this was reflected in the overall value for Weeks 1-13 (↓17%, ↓22% and ↓12%). Food utilization for males treated at 1700 ppm was less efficient than control for the period 5-8 weeks only (↓19%). Females treated at 7000 ppm showed a slightly decreased food utilization profile throughout Weeks 1-13, with statistical significance being achieved for Weeks 9-13 (↓64%), and as an overall result for Weeks 1-13 (↓33%) . The isolated difference in food utilization for females treated at 70 ppm during Weeks 9-13 (↓44%), was considered not to be related to treatment in the absence of a similar difference in females treated at 1700 ppm.

Table 4: Intergroup comparison of food utilization (g) - selected timepoints

	Dietary Concentration of bicyclopyrone (ppm)							
	Males				Females			
weeks	0	70	1700	7000	0	70	1700	7000
1-4	3.5 ± 1.1	3.8 ± 1.1	3.5 ± 1.2	2.9** ± 1.0 (↓17%)	3.5 ± 1.0	3.3 ± 0.5	3.2 ± 1.1	2.9 ± 0.8
5-8	2.7 ± 0.9	2.4 ± 0.9	2.2** ± 1.0 (↓19%)	2.1**± 0.8 (↓22%)	2.6 ± 1.2	2.8 ± 1.0	2.1 ± 1.0	2.2 ± 0.6
9-13	1.6 ± 0.9	2.0 ± 0.8	1.5 ± 1.1	1.7 ± 1.0	2.5 ± 0.9	1.4** ± 1.1 (↓44%)	2.2 ± 0.9	0.9** ± 0.8 (↓64%)
1-13	2.5 ± 0.7	2.7 ± 0.7	2.3 ± 0.8	2.2* ± 0.6 (↓12%)	2.8 ± 0.7	2.4 ± 0.7	2.4 ± 0.7	1.9** ± 0.4 (↓33%)

Data were taken from pages 96-97 of the study report

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Dose rates (based on nominal dietary levels of bicyclopyrone) were calculated in terms of mg bicyclopyrone/ kg body weight. Mean values are shown in Table 5.

Table 5: Mean dose received (mg/kg/day)

bicyclopyrone (ppm)	70	1700	7000
Males	8.7	233	940
Females	9.2	242	1027

Data were taken from pages 35-70 of the study report

Water consumption: There were no observable differences between treated and control groups.

Haematology: There were no differences in haematology parameters which were considered to be attributable to treatment with bicyclopyrone.

Higher, although not statistically significant, mean white cell counts, were noted in males treated at 70 ppm. This reflected an abnormally high value for one animal, which was diagnosed with malignant lymphoma.

Mean white cell counts in females treated at 7000 ppm were statistically significantly higher than control. This reflected higher, although not statistically significant, values for large unclassified cells, lymphocytes and neutrophils; however, it is considered that the higher values at 7000 ppm were the result of three individual animals two with lymphoid hyperplasia, and one with malignant lymphoma).

Sacrifice and pathology:
Macroscopic findings: A small number of lesions were observed, none of which were considered to be related to treatment.

Organ weights: Liver weights, adjusted for terminal body weight, were statistically significantly increased in all male treated groups (↑25%-↑28%), where no clear dose response was apparent, and in females at 1700 and 7000 ppm (↑20%-↑31%). Changes in absolute liver weights correlated with adjusted liver weights in all cases. See table 6.

Other slight variances from the control organ weights were noted in either sex, some of which were seen to be statistically significant. However, due to a lack of corroborating data, evidence of a dose related response or consistency between sexes, it is considered that these findings were not treatment related.

Table 6: Intergroup comparison of liver weights (g)

	Dietary concentration of bicyclopyrone (ppm)							
	Males				Females			
	0	70	1700	7000	0	70	1700	7000
absolute	3.11 ± 0.74	3.90** ± 1.46 (↑25%)	3.52 ± 0.99 (↑13%)	3.76* ± 1.09 (↑21%)	2.41 ± 0.46	2.59 ± 0.56	2.79** ± 0.74 (↑16%)	2.96** ± 0.75 (↑23%)
adjusted	3.03 ± 0.16	3.79** ± 0.16 (↑25%)	3.59* ± 0.17 (↑18%)	3.89** ± 0.16 (↑28%)	2.37 ± 0.08	2.45 ± 0.08	2.85** ± 0.08 (↑20%)	3.11** ± 0.09 (↑31%)

Data were taken from pages 100-103 of the study report

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Microscopic findings: A small number of spontaneous lesions were observed, none of which was related to treatment.

Non-neoplastic: Data for non-neoplastic lesions are presented on table 7. There was a statistically significant increase in the incidence of centrilobular hypertrophy ($p<0.001$) in the liver of males receiving 7000 ppm compared to controls. This finding was noted in 36/50 males receiving 7000 ppm where all gradings were mild, compared to 0/50 in controls (72% vs. 0%).

There was a statistically significantly lower incidence of adrenal subcapsular cell hyperplasia in both sexes at 7000 ppm (generally where the grading was mild), compared with controls ($p<0.05$).

Table 7: Intergroup comparison of selected non-neoplastic histopathological findings

Finding	Dietary concentration of bicyclopyrone (ppm)							
	Males				Females			
	0	70	1700	7000	0	70	1700	7000
Liver centrilobular hypertrophy	0/50	0/50	0/50	36/50*** (72%)	0/49	0/50	0/50	0/50
Adrenal subcapsular cell hyperplasia	24/50 (48%)	19/50 (38%)	15/50 (30%)	12/49* (25%)	40/49 (82%)	35/50 (70%)	33/50* (66%)	29/50* (58%)

Data were taken from pages 127-103 of the study report
* Statistically significant difference from control group mean, $p<0.05$
*** Statistically significant difference from control group mean, $p<0.001$

Neoplastic: There were no tumors considered related to treatment with bicyclopyrone. There was no difference in the overall number of tumors, the number of tumor bearing animals or the time to onset of any tumor type.

Table 8: Intergroup comparison of bronchio-alveolar histopathological findings male mice

Finding	Dietary concentration of bicyclopyrone (ppm)			
	Males			
	0	70	1700	7000
Number of mice examined	50	50	50	50
Bronchio-alveolar carcinoma (M)	2	3	3	4
Bronchio-alveolar adenoma (B)	9 (18%)	13	13	18 (36%)
Brochio-alveolar hyperplasia				
Minimal	0	0	0	1
Mild	2	1	1	1
Total	2	1	1	2

Data were taken from page 195 of the study report

The incidence of bronchiole-alveolar adenoma in the lung was numerically higher in males receiving 7000 ppm compared to concurrent controls (Table 8). A Peto trend test revealed a statistically significant increasing trend in the incidence of lung bronchiolo-alveloar adenoma [B] in males ($p=0.034$). When the high dose group (group 4) was excluded from the analysis, the test for increasing trend was no longer statistically significant ($p=0.24$). This incidence at 7000 ppm in males was not statistically significantly different from the control by pair-wise comparison. There were no differences in female mice. The incidence of benign tumors in the male lung at the limit dose of 7000 ppm was not accompanied by any other histopathological changes in the lungs and was similar to the incidence seen in control groups from studies being conducted concurrently. See table 9.

Table 9: Historical Control for Lung Bronchiolo-Alveolar Tumors and Hyperplasia: Crl:CD-1 Mice

Study identifier	Study start	Route of administration	Number of lungs examined	Males		
				Hyperplasia	Adenoma	Carcinoma
283	Sep 2007	Dietary	50	3	12	5
613	Oct 2007	Dietary	50	0	14	1
629	Sep 2007	Dietary	50	0	13	1
575	Sep 2007	Dietary	50	0	15	4
Total			**200**	**3**	**54**	**11**
			Range	**0-6%**	**24–30%**	**2-10%**

Data were taken from page 31 of the study report

This numerically higher incidence of bronchiole-alveolar adenoma in males at 7000 ppm was considered not to be treatment related.

INVESTIGATOR'S CONCLUSIONS

Dietary administration of bicyclopyrone at 0, 70, 1700 and 7000 ppm, for a period of at least 80 weeks, was associated with decreases in body weight and body weight gain and less efficient food in males and females treated at 7000 ppm.

There were no tumours considered to be related to treatment with bicyclopyrone. There was no difference in the overall number of tumours, the number of tumour bearing animals or the time to onset of any tumour type.

The No Observed Adverse Effect Level (NOAEL) for bicyclopyrone was 1700 ppm (233 and 242 mg/kg/day) in males and females respectively.

REVIEWER COMMENTS

The purpose of this study was to evaluate the carcinogenic potentiatal of pyrifluquinazon in mice for 18 months through oral exposure. Bicyclopyrone (NOA449280, purity 94.5% w/w) was administered to groups of 50 male and 50 female CD-1 mice in the diet at dose levels of 0, 70, 1700 and 7000 ppm (equivalent to 0, 8.7 / 9.2, 233 / 242, 940 / 1027 mg/kg bw/day for males / females respectively) for a period of at least 80 weeks.

There were no treatment related clinical observations and no effects on mortality at any dose. At all doses, there were changes at 70 and 1700 ppm, such as increased absolute and adjusted liver weights which were considered adaptive.

At 7000 ppm bicyclopyrone, there was a statistically significant decrease in the absolute body weights in females (↓9-12%).

EPA's Cancer Assessment Review Committee (CARC) concluded that in male mice, there was a trend and pairwise significance at the high dose for adenomas and combined tumors. However, these tumors were determined to be "not treatment related" based on the following considerations:

- Only a marginal increase in adenomas was observed in comparison to the

concurrent control. The incidence for lung adenomas in the concurrent controls was slightly lower than the historical control range for this tumor at 80 weeks which resulted in the marginal increase of adenomas;
- There was no increases in carcinomas (*i.e.* no malignant progression);
- There were no corroborative precursor lesions (i.e. hyperplasia or other non-neoplastic lesions) observed at this dose;
- Lung tumors are a common background tumor in mice of this age; and
- Lung tumors were not seen in pesticide chemicals of this class (no structural activity relationship (SAR) support).

Regarding the adequacy of dosing, the highest dose tested was the Limit Dose (7000 ppm or 1000 mg/kg/day) in both sexes and was considered to be adequate and not excessive for assessing carcinogenicity.

Based upon the effects in this study, the LOAEL is 7000 ppm (1027 mg/kg/day [F]) based upon decreased absolute body weights in females. The NOAEL is 1700 ppm (242 mg/kg/day [F]). The NOAEL for males is 7000 ppm (940 mg/kg/day). The LOAEL for males was not established.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirements (OPPTS 870.4200) for an oncogenicity study in the mice. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Robertson B, 2012)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/18/15
EPA Reviewer: Greg Akerman, Ph.D. **Signature:** Greg Akerman
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/18/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Carcinogenicity and combined 52 week toxicity study – Rat (feeding)

OECD 453 (2009): OPPTS 870.4300 (1998): EU Directive 96/64/EEC B.33 (2001): JMAFF No. 12-Nohsan-8147 (2000)

TEST MATERIAL (PURITY): NOA449280 (94.5% purity)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[[2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]-; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one; bicyclopyrone, SYN449280.

CITATION: Robertson B and Perry C, 2012. NOA449280: 104 week rat dietary carcinogenicity study with combined 52 week toxicity study. Charles River, Tranent, Edinburgh, EH33 2NE, UK. Laboratory Report No. 30197, 31 August 2012. (Syngenta File No.NOA449280_11302). MRID 47841985

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a combined 52 week chronic/104 week rat dietary carcinogenicity study (MRID #47841985), bicyclopyrone (94.5% purity) was administered to groups of Han Wistar (CrL:WI(Han)) rats in the diet. Five groups of 52 male and 52 female Han Wistar rats were assigned to the Carcinogenicity study and dosed with diets containing 0, 5, 500, 2500 or 5000 ppm bicyclopyrone for at least 104 consecutive weeks. In addition, a chronic toxicity study comprising a further 5 groups of 12 males and 12 females was included and dosed in an identical fashion for a period of 52 consecutive weeks. The equivalent doses for the carcinogenicity phase of the study were 0, 0.28/0.35, 28.4/35.8, 141/178 and 280/368 mg/kg/day (M/F). The equivalent doses for the chronic toxicity phase of the study were 0, 0.32/0.39, 32.6/41.6, 166/204 and 335/404 mg/kg/day (M/F). Since the achieved doses are similar between the two phases, the doses from the carcinogenicity phase will be used for risk assessment purposes.

Due to observation of severe eye lesions, corneal opacity and damage, 5000 ppm male animals were fed blank control diet over a 9 day period during Weeks 4 and 5. Dosing recommenced after this period and was continuous to the end of the study.

The following were assessed at pre-determined intervals from pre-trial until study completion from carcinogenicity and chronic toxicity study animals: clinical observations, body weight, food consumption, haematology, coagulation and clinical chemistry. Additionally, selected carcinogenicity study animals had samples taken for urinalysis at predetermined intervals and all underwent ophthalmoscopy examinations prior to initiation of dosing and at weeks 50 and 102. Toxicity study animals received a detailed functional observation battery assessment once during treatment (weeks 51/52).

All surviving Carcinogenicity and Toxicity study animals were terminated and subjected to a detailed necropsy examination with a comprehensive histological evaluation after the completion of 104 or 52 weeks of treatment respectively.

The effects are as follows:

There were no statistically significant differences in mortality between the controls and any groups treated with bicyclopyrone.

At 5 ppm bicyclopyrone, there was a 2-6% increase in the incidence of opaque eyes and corneal damage in both sexes compared to the control group (0-2%). At 104 weeks in males, there was an increased incidence of thyroid follicular hyperplasia in males (19%) compared to the control group (4%). There was also an increase in the incidence of chronic progressive nephropathy in the kidneys of males (63%) compared to the control group (33%).

At 500 ppm bicyclopyrone, there was a significant increase in the incidence of opaque eyes and corneal damage in both sexes (98-100%) compared to controls (0-2%). There was an increase in the incidence of eye keratitis (88-100% for males and 87-100% for females) and the regenerative corneal hyperplasia (88-100% for males and 42-92% for females) from 52 weeks to 104 weeks compared to the control group (2%). In males, there was an increased incidence of thyroid follicular hypertrophy (75%) at 52 weeks compared to the control group (0%). At 104 weeks in males, there was an increased incidence of thyroid follicular hyperplasia (23%) compared to the control group (19%). This effect occurred in females as well but there was no dose response. There was also an increase in the incidence of chronic progressive nephropathy in the kidneys of males (75%) compared to the control group (33%). In males, there was an increased incidence of squamous cell carcinoma and papilloma (4% and 2%) compared to the control group (0%).

At 2500 ppm bicyclopyrone, there was a significant increase in the incidence of opaque eyes and corneal damage in both sexes compared to controls (98-100%) compared to the control group (0-2%). Decreases in absolute body weights for females were transiently statistically significant through the study (↓5-10%). Relative to the control group, there was a minor decrease in the absolute brain weights of males and females (↓3-7%), and heart weights of females (↓7%). There was an increase in the incidence of eye keratitis (83% for males and 87-92% for females) and regenerative corneal hyperplasia (58-63% for males and 58% for females) from 52 weeks to 104 weeks compared to the control group (2%). In males, there was an increased incidence of thyroid follicular hypertrophy (83%) at 52 weeks compared to

the control group (0%). At 104 weeks in males, there was an increased incidence of thyroid follicular hyperplasia (23%) compared to the control group (4%). There was also an increase in the incidence of chronic progressive nephropathy in the kidneys of males (77%) compared to the control group (33%). There was a statistically significant increase in the incidence of acinar cell atrophy in the pancreas of male animals (50%) compared to the control group (27%). In males, there was an increased incidence of squamous cell carcinoma and papilloma (4% and 2%) compared to the control group (0%).

At 5000 ppm bicyclopyrone, there was a significant increase in the incidence of opaque eyes and corneal damage in both sexes (98-100%) compared to the control group (0-2%). Relative to the control group, in both sexes there were significantly lower body weights (↓5-16% for males and ↓5-20% for females). There were also minor changes in food consumption and utilization. There was an increase in the incidence of eye keratitis (88-100% for males and 73-92% for females) and the regenerative corneal hyperplasia (71-100% for males and 35-75% for females) from 52 weeks to 104 weeks compared to the control group (2%). In males, there was an increased incidence of thyroid follicular hypertrophy (66%) at 52 weeks compared to the control group (0%). At 104 weeks in males, there was an increased incidence of thyroid follicular hyperplasia (33%) compared to the control group (4%). There was also an increase in the incidence of chronic progressive nephropathy in the kidneys of males (69%) at 104 weeks compared to the control group (33%). There was a statistically significant increase in the incidence of acinar cell atrophy in the pancreas of male animals (58%) compared to the control group (27%). In males, there was an increased incidence of squamous cell carcinoma and papilloma (4% and 6%) compared to the control group (0%).

The corneal tumors seen in males rats are associated with and likely attributable to significant damage to and regenerative hyperplasia of the cornea seen during the course of the carcinogenicity study with bicyclopyrone at concentrations of 500 ppm and above. The identified mode of action of HPPD inhibiting herbicides results in significantly elevated plasma tyrosine in rats, particularly males. EPA's Cancer Assessment Review Committee determined that in male rats, there was a dose-dependent increase in corneal tumors which were considered treatment related (Rowland et al., September 10, 2014, TXR #0057011). The doses tested were considered to be adequate and not excessive, for assessing carcinogenicity in both sexes. This was based upon increases in corneal opacity, decreased absolute body weights in both sexes at the high dose, and an increased incidence of regenerative corneal hyperplasia in both sexes.

Based upon the effects in this study, the LOAEL for systemic toxicity is 5 ppm (0.28/0.35 mg/kg/day [M/F]) based on a dose dependent increase in the incidence of opaque eyes and corneal damage in both sexes compared to controls, an increased incidence of thyroid follicular hyperplasia in males, and an increased incidence of chronic progressive nephropathy in the kidneys of males. The NOAEL was not established.

This study is classified as totally reliable **(acceptable/guideline)** as a combined chronic/carcinogenicity study in rats (OPPTS 870.4300; OECD 451). EPA, PMRA, and AMPVA agree on the regulatory decision and classification for this study.

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, Flagging and Quality Assurance statements were provided.

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Technical, solid beige powder
Lot/Batch number:	SEZ3AP006/MILLED
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Stable (stored at a temperature < 30°C; light protected, dry)

Structure:

Vehicle and/or positive control: The test substance was administered via Rat and Mouse (modified) No. 1 Diet SQC Expanded (Ground) (Special Diets Services Limited, 1 Stepfield, Witham, Essex, UK)).

Test Animals:

Species	Rat
Strain	Han Wistar (CrL:WI(Han))
Age/weight at dosing	Approximately 6 weeks / 104-184 g (males), 96-155 g (females)
Source	Charles River UK Limited, Margate, Kent, UK
Housing	Up to 4 per cage by sex and dose group, in suspended polycarbonate cages (overall dimensions 59 x 38.5 x 20 cm) with stainless steel grid tops and food hopper.
Acclimatisation period	13 days
Diet	Rat and Mouse (modified) No. 1 Diet SQC Expanded (Ground) (Special Diets Services Limited, 1 Stepfield, Witham, Essex, UK) *ad libitum*
Water	Mains water *ad libitum*
Environmental conditions	Temperature: 9.41-23.69°C (target range 19-23°C) Humidity: 13.87-85.06% (target range 40-70%) Air changes: Minimum of 15 air changes per hour Photoperiod: 12 hrs dark / 12 hrs light

In-life dates: Start: 30 April 2007 End: 20 October 2009

Study Design and Methods: In a 104 week rat dietary carcinogenicity study with combined 52 week chronic toxicity study, NOA449280 (94.5% purity) was administered to groups of Han Wistar (CrL:WI(Han)) rats in the diet.

Carcinogenicity study animals (52/sex/dose) were dosed continuously by the diet for at least 104 weeks and chronic toxicity study animals (12/sex/dose) for at least 52 weeks with the exception of 5000 ppm males where the animals were removed from treatment and given

blank diet on 25 May 2007 due to eye lesions. Dosing recommenced for these animals on the 04 June 2007.

Animal assignment: On arrival from the suppliers, the animals were introduced to cages on racks. Cages were racked by treatment group and vertically throughout the rack. Each group in the carcinogenicity study was housed on a separate rack. In the chronic toxicity study, control animals were housed on the same rack as treated groups. Each month, from the commencement of pretrial, each column of cages on a rack was moved one position to the right. During pretrial, group mean body weights were checked to ensure that all groups had a similar body weight for each sex and were all found to be within a 20% limit of variation. Animals were allocated to dose groups as in the table below:

Table 1: Study design

Test group	Dietary concentration (ppm)	Animal numbers			
		Carcinogenicity study		Chronic toxicity study	
		males	females	males	females
Control	0	1-52	261-312	521-532	581-592
Low	5	53-104	313-354	533-544	593-604
Intermediate 1	500	105-156	365-416	545-556	605-616
Intermediate 2	2500	157-208	417-468	557-568	617-628
High	5000	209-260	469-520	569-580	629-640

*Table was taken from page 22 of the study report

Diet preparation and analysis: Control, 500, 2500 and 5000 ppm diets were made and dispensed weekly for administration to the animals. 5 ppm diets were made weekly, and stored frozen at -20°C until required. Diet preparations were made as a serial dilution from a stock of the high dose level of 5000 ppm. The stock was prepared by mixing test item with the required amount of untreated control diet in an automated mortar and pestle and ground for 5 min. The premix was then blended with the required amount of untreated diet and mixed for 20 minutes in a diet mixer (Winkworth). The diets at the lower concentrations (5, 500, 2500 and an intermediate level of 100 ppm) were prepared as a serial dilution from the higher concentration group by adding an appropriate amount of untreated diet. Diets were mixed for 20 minutes in a Winkworth change drum mixer. The diets were stored at ambient room temperature (except for the 5 ppm dose up until week 12 which was stored -20ºC in the dark).

Prior to study commencement, stability data was generated by Charles River, Edinburgh for diet preparations stored at -20°C for 15 days and for 7 days (500-5000 ppm) or 1 day (5 ppm) at ambient laboratory temperature. During the study, triplicate samples (3 x 50 g) were taken from each diet (including control) at approximately 3 monthly intervals immediately after preparation and analysed for concentration and homogeneity.

Concentration analysis results: Analysed concentrations of test item within the diet were generally found to be within ±8.2% of the theoretical concentrations.

Homogeneity results: The Week 1 low dose group (5 ppm) was out of the acceptable criteria for homogeneity (CV ≤37%) and was re-analyzed. Subsequent analyses for all samples were with the acceptable range (CV <12.3%).

Stability results: Stability analyses revealed differences in concentrations ranging from 2.4-13.6% of the theoretical, with coefficients of variation ranging from 0.3-8.4%. Deviations were isolated and were considered to be minimal and not to have affected the outcome or integrity of the study. Bicyclopyrone was not detected in the control diet.

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable, provided that the cited stability study did indicate that the test compound was stable under conditions of the study.

Observations: All animals were checked twice per day for signs of viability. Once each week all animals received a detailed clinical examination, including appearance, movement and behaviour patterns, skin and hair condition, eyes and mucous membranes, respiration and excreta.

Body weight: The body weight of each rat was recorded once weekly during pre-trial up until Week 14 of treatment, and once every 2 weeks from week 16 up until the end of treatment.

Food consumption and test substance intake: The quantity of food consumed by each cage of animals was measured and recorded once weekly during pre-trial up until week 14 of treatment, and once every 4 weeks from week 16 up until the end of treatment. Food utilization was calculated for weeks 1-4, 5-8, 9-13 and 1-13, according to the following formula:

(Cage mean weight gain x 100) / cage total food consumption.

The amount of test item ingested was calculated at regular intervals during treatment using the following formula:

$$\text{Achieved intake (mg/kg/day)} = \frac{\text{Nominal Concentration (ppm)} \times \text{Food Consumption (g/day)}}{\text{Mid-point Body Weight (g)}}$$

Water consumption: Water consumption was qualitatively monitored by visual inspection of the water bottles on a weekly basis throughout the study.

Ophthalmoscopic examination: Eyes were examined using an indirect ophthalmoscope following application of a mydriatic agent (1% Tropicamide, Mydriacyl®). The cornea, anterior chamber, iris, lens, posterior chamber, retina and vessels of the optic disc were examined from all Carcinogenicity animals during pre-trial and weeks 50 and 102.

Functional observation battery: Once during the treatment period (week 51/52) a more detailed examination was made of all chronic toxicity study animals. The examinations were made by a technician not involved in the dosing procedures or in the collection of body weights and food consumption data, and were performed at an approximately standardised time of day. Prior to the independent technician entering the room, standard cage cards were removed and only neurotoxicity cards were shown. The assessor was then allowed to enter the room. Three animals from each cage had their tail marked for identification purposes. The following were assessed:

Cage side observations: Prostration, lethargy, writhing, circling, breathing abnormalities, gait abnormalities, tremor, fasciculation, convulsions, biting (of cage components or self mutilating), vocalisations, piloerection, ease of removal from the cage, body temperature (taken directly by from the implanted electronic chip and recorded), condition of the eyes (checked for: pupillary function, miosis, mydriasis, exophthalmos, encrustation, lachrymation), condition of the coat, presence of salivation, overall ease of handling.

Observations in a standardised area (2 min observation): Latency (time to first locomotory movement), level of mobility, rearing, grooming, urination/defecation, arousal (level of alertness), posture, tremor/convulsions, vocalisation, piloerection, palpebral closure, gait abnormalities, stereotypy (excessive repetition of behaviours) and/or unusual behaviours.

Functional Tests: Once during the treatment period (week 51/52), the following additional functional tests were performed: Reaction to sudden sound (click above the head), reaction to touch on the rump with a blunt probe, grip strength, pain perception, landing foot splay.

Motor activity: Each animal was placed in an individual monitoring cage, scanned by a motion sensor utilising infra-red pyroelectric detectors. Movement was detected in 3 dimensions anywhere in the cage, and was differentiated into large and small movements. Each animal was monitored for one session with movement recording at 5-minute intervals, and each session was run for at least 1 hour (between 71–84 minutes).

Clinical pathology: Blood samples for haematology (0.5 mL, into EDTA tubes), coagulation (0.5 mL, into 0.045 mL trisodium citrate tubes) and clinical chemistry (1.5 mL, into lithium heparin tubes) were obtained, *via* the tail vein and without anaesthesia, from 13 males and 13 females per group from the carcinogenicity study at weeks 14, 27, 53 and 79, all surviving carcinogenicity study animals during weeks 104, and all surviving Toxicity study animals during Week 52. The animals were not deprived of food overnight prior to sampling.

Haematology and coagulation: The following parameters were examined:

haemoglobin
haematocrit
red blood cell count
mean cell volume
mean cell haemoglobin
prothrombin time
mean cell haemoglobin concentration
platelet count
total white cell count
differential white cell count
activated partial thromboplastin time

Clinical chemistry: The following parameters were examined:

urea
creatinine
glucose
albumin
total protein
cholesterol
triglycerides
total bilirubin
creatine phosphokinase
globulin
alkaline phosphatase activity
aspartate aminotransferase activity
alanine aminotransferase activity
gamma-glutamyl transferase activity
calcium
phosphate
sodium
potassium
chloride
AG ratio

Urinalysis: Urine samples were collected over a 4 h period from 13 male and 13 female carcinogenicity study animals per group during Weeks 13, 26, 52, 78 and 103. The animals were housed individually in metabolism cages and were deprived of food and water. The following parameters were evaluated:

volume
colour
specific gravity
pH
urobilinogen
microscopy of spun deposit
glucose
ketones
protein
bilirubin
blood pigments

Investigations *post mortem:* After at least 52 or 104 weeks of treatment all surviving animals were killed in random order by exposure to carbon dioxide and had their terminal body weight recorded, followed by exsanguination.

Macroscopic examination: All animals were examined *post mortem*. This consisted of a complete external and internal examination which included body orifices (ears, nostrils, mouth, anus, vulva) and cranial, thoracic and abdominal organs and tissues.

Organ weights: From all chronic toxicity and carcinogenicity animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

adrenal glands
brain
epididymides
heart
kidneys
ovaries
spleen
testes
liver
uterus (with oviducts)

Paired organs were weighed together.

Tissue submission: The following tissues from all chronic toxicity and carcinogenicity animals, surviving to scheduled termination, were examined *in situ*, removed and processed top paraffin wax blocks, stained with haematoxylin and eosin and examined histopathologically:

gross lesions including masses (with lymph nodes local to masses)
adrenal gland
aortic arch
brain (forebrain, midbrain, cerebellum, pons)
bone marrow (femur)
caecum
colon
duodenum
epididymis
eyes (including optic nerve)
oesophagus
ovary
oviduct
Peyer's patches
pancreas
parathyroid gland
pharynx
pituitary gland
prostate gland
rectum

Harderian gland
heart
ileum
jejunum
kidney
lachymal gland
larynx
liver
lung
lymph node – mesenteric and submandibular
thigh muscle
tongue
uterus
vagina
salivary gland
seminal vesicle
spinal cord (cervical, mid thoracic, lumbar)
skin and mammary gland
spleen
sternum
sciatic nerve
stomach
testis
thymus
thyroid gland
trachea
urinary bladder

Microscopic examination: All processed tissues were 4-6 µm thick and were examined by light microscopy.

Statistics: Body weight, cumulative body weight gain, food consumption, food utilization, haematology, coagulation, clinical chemistry, quantitative urinalysis values, quantitative functional observation battery measurements, motor activity and organ weight data were analysed using a parametric ANOVA and pairwise comparisons made using the Dunnett's t-test. The following pairwise comparisons were performed: Control Group vs Low Dose, Control Group vs Intermediate Dose, Control Group vs High Dose. Organ weights were also analysed by analysis of covariance (ANCOVA) using terminal kill body weight as covariate. Kaplan-Meier survival estimates were calculated separately for each sex and treatment group. Histological incidence data and pairwise comparisons of the incidence of tumor and histological lesions were made using Fisher's Exact test. Further analysis was performed using Peto's time adjusted methods. Methods used for the age-adjusted analysis of fatal and non-fatal tumors were based on the IARC guidelines.

RESULTS AND DISCUSSION

Mortality: There was no statistically significant difference in mortality between the control and any other groups for males or females.

Clinical observations: Almost all males and females treated at 500 ppm and above were noted to have opaque eyes and corneal damage (≥98% of animals in these groups). The distribution was equal between both eyes. This finding was generally seen from approximately Week 4 until the end of the treatment period. See table 2.

Table 2: Intergroup comparison of the incidence of opaque eyes and corneal damage

Finding	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
Eyes opaque										
Number of animals	1 (2%)	3 (6%)	51 (98%)	51 (98%)	51 (98%)	0	1 (2%)	52 (100%)	52 (100%)	50 (100%)

Days from - to	408-737	387-737	24-737	23-737	23-737		640-710	24-738	24-738	24-738
Corneal damage										
Number of animals	0	1 (2%)	51 (98%)	51 (98%)	51 (98%)	0	0	51 (98%)	52 (100%)	50 (96%)
Days from-to		352-359	24-737	24-737	24-737			24-738	24-738	24-738

Data were taken from page 43 of the study report

Body weight and weight gain: Significantly lower absolute body weight was seen in both sexes throughout the study at 2500 (↓5-7% for males and ↓4-10% for females) and 5000 ppm (↓5-10% for males and ↓6-20% for females). Decreases for males at 500 ppm were not statistically significant. See table 3.

Table 3: Mean intergroup comparison of absolute bodyweights (g) by week

	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
Week*	**0**	**5**	**500**	**2500**	**5000**	**0**	**5**	**500**	**2500**	**5000**
Pre-Test (Week 0)	143.8 ± 14.1	139.3 ± 16.2	137.7 ± 17.3	147.3 ± 16.1	146.1 ± 16.1	122.5 ± 12.4	125.5 ± 12.7	127.8* ± 9.9 (↑4%)	125.4* ± 12.0 (↑2%)	128.8** ± 10.5 (↑5%)
Week 2	213.4 ± 23.3	223.3 ± 21.0	201.0 ± 27.7	213.7 ± 18.1	203.0* ± 23.0 (↓5%)	158.2 ± 14.9	168.9** ± 14.7 (↑6%)	164.7* ± 11.7 (↑4%)	159.9 ± 12.1	160.1 ± 12.5
Week 7	332.5 ± 32.9	340.5 ± 28.7	320.3 ± 28.9	312.7** ± 24.0 (↓6%)	299.9** ± 30.8 (↓10%)	216.2 ± 17.7	220.3 ± 18.7	215.5 ± 15.5	208.6* ± 16.6 (↓4%)	203.9* ± 14.5 (↓6%)
Week 13	377.6 ± 38.9	386.3 ± 35.6	365.6 ± 34.1	357.1** ± 33.8 (↓5%)	345.6** ± 36.9 (↓8%)	237.1 ± 18.8	241.1 ± 21.5	236.2 ± 16.9	229.9 ± 18.8	225.3** ± 16.8 (↓5%)
Week 26	440.4 ± 42.1	448.9 ± 44.0	422.5 ± 41.7	412.0** ± 37.9 (↓6%)	403.7** ± 44.8 (↓8%)	261.9 ± 20.7	261.7 ± 22.6	257.4 ± 21.1	250.2** ± 20.4 (↓4%)	243.4** ± 17.2 (↓7%)
Week 52	529.4 ± 50.8	540.8 ± 59.9	511.7 ± 56.5	499.4** ± 52.8 (↓6%)	487.6** ± 47.0 (↓8%)	301.9 ± 40.0	306.2 ± 37.7	298.6 ± 40.6	289.7 ± 39.0	267.4** ± 23.0 (↓11%)
Week 78	583.2 ± 67.6	603.5 ± 80.8	574.4 ± 70.1	551.1 ± 64.9	541.6* ± 56.8 (↓7%)	366.9 ± 53.2	377.1 ± 54.5	362.2 ± 59.4	343.4 ± 54.8	303.5** ± 34.3 (↓17%)
Week 96	612.3 ± 69.3	627.6 ± 91.7	594.3 ± 74.4	568.8* ± 59.0 (↓7%)	565.9* ± 59.0 (↓8%)	403.6 ± 52.6	399.2 ± 60.8	397.3 ± 64.6	366.1* ± 58.8 (↓9%)	323.8** ± 39.4 (↓20%)
Week 104	610.8 ± 74.9	635.8 ± 89.8	593.0 ± 74.2	569.6* ± 63.4 (↓7%)	561.5* ± 61.5 (↓8%)	406.5 ± 59.6	408.8 ± 69.5	405.9 ± 67.2	367.0* ± 58.9 (↓10%)	328.4** ± 45.2 (↓19%)

Data were taken from pages 88-97 of the study report

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Significantly lower body weight gains were seen in both sexes throughout the study at 2500 (↓8-14% for males and ↓9-20% for females) and 5000 ppm (↓10-26% for males and ↓16-37% for females). In 500 ppm females there was a decrease in body weight gains from weeks 0-1 (↓18%) and from weeks 0-26 (↓7%). See table 4.

Table 4: Intergroup comparison of bodyweight gain – carcinogenicity and toxicity studies combined (g) - selected weeks

Week	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
0-1	39 ± 8.0	43* ± 4.7 (↑10%)	43* ± 5.9 (↑10%)	38 ± 4.6	29** ± 10.4 (↓26%)	25 ± 4.5	24 ± 5.2	21** ± 4.9 (↓18%)	20** ± 5.0 (↓20%)	16**± 4.2 (↓37%)
0-4	132 ± 17.9	145** ± 15.1 (↑10%)	131 ± 18.5	115** ± 16.1 (↓14%)	98** ± 17.5 (↓26%)	69 ± 10.1	72 ± 13.6	64 ± 10.2	59**± 10.7 (↓14%)	53** ± 7.7 (↓23%)
0-13	234 ± 33.4	247 ± 32.2	228 ± 32.3	210** ± 33.0 (↓10%)	199** ± 29.0 (↓15%)	115 ± 14.8	116 ± 18.8	109 ± 13.8	105** ± 15.6 (↓9%)	97** ± 12.2 (↓16%)
0-26	297 ± 37.1	310 ± 41.4	285± 39.7	265** ± 37.3 (↓11%)	258** ± 37.2 (↓13%)	140 ± 16.8	136 ± 20.3	130** ± 17.8 (↓7%)	125**± 16.9 (↓11%)	114** ± 12.6 (↓18%)
0-52	385 ± 48.2	401± 57.8	375 ± 54.5	352**± 53.1 (↓8%)	341** ± 41.7 (↓11%)	180 ± 35.1	181 ± 35.9	171 ± 38.1	164** ± 37.0 (↓9%)	139** ± 19.3 (↓23%)
0-78	439 ± 66.1	462 ± 79.7	437 ± 66.7	403** ± 64.5 (↓8%)	394** ± 51.9 (↓10%)	247 ± 48.0	253 ± 50.9	235 ± 57.1	219** ± 50.7 (↓11%)	175** ± 30.6 (↓29%)
0-104	468 ± 74.2	493 ± 88.1	457 ± 73.3	422** ± 63.8 (↓10%)	414** ± 57.0 (↓11%)	287 ± 54.0	285 ± 65.4	280 ± 66.0	244** ± 55.0 (↓15%)	201** ± 41.5 (↓30%)

Data were taken from pages 98-107 of the study report

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Food consumption, utilization and compound intake: Decreases in food consumption and food utilization were seen in 2500 and 5000 ppm males and females, with decreases in food utilization observed sporadically in 500 ppm females. See tables 5, 6, and 7.

Table 5: Intergroup comparison of food consumption (g/animal/day) – carcinogenicity and toxicity studies combined (g) - selected weeks

Week	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
1	20.8 ± 1.6	22.0* ± 1.1 (↑6%)	21.6 ± 1.1	20.6 ± 1.3	19.9 ± 1.6	17.1 ± 0.7	17.5 ± 1.8	16.8± 1.2	16.6 ± 1.4	16.4 ± 2.3
3	24.0 ± 1.6	24.1 ± 1.4	22.9 ± 2.7	19.8**± 4.3 (↓18%)	17.7** ± 4.2 (↓26%)	16.5± 2.6	17.7 ± 0.8	15.8 ± 2.5	14.6* ± 2.5 (↓12%)	15.2 ± 1.9
12	20.9 ± 1.4	21.4 ± 1.4	21.2 ± 1.0	21.1 ± 1.2	20.1 ± 1.4	16.5 ± 0.8	17.1 ± 1.0	17.4* ± 1.0	16.7 ± 1.1	16.2± 1.0
16	22.2 ± 1.1	22.8 ± 1.4	23.1 ± 1.2	22.3 ± 0.9	21.4 ± 1.0	18.2 ± 1.0	17.8 ± 1.1	18.8 ± 1.1	18.0 ± 1.0	17.7 ± 1.1
32	22.4 ± 1.3	22.4 ± 1.4	21.8 ± 1.0	21.1**± 0.9 (↓6%)	20.7** ± 1.0 (↓8%)	18.0 ± 1.0	17.7 ± 0.8	18.3 ± 1.3	17.7 ± 1.0	16.6** ± 1.0 (↓8%)

60	22.5 ± 1.0	22.1 ± 1.1	20.9** ± 1.1 (↓7%)	20.8** ± 1.2 (↓8%)	20.1** ± 1.1 (↓11%)	18.7 ± 1.5	19.3 ± 1.4	18.9 ± 1.4	18.4 ± 1.6	17.4 ± 1.3
80	22.3 ± 2.0	22.2 ± 1.0	21.4 ± 1.0	21.0* ± 1.4	21.0 ± 1.8	19.1 ± 1.5	19.7 ± 1.3	19.3 ± 1.8	18.3 ± 1.1	17.5* ± 1.5 (↓8%)
104	21.3 ± 2.7	22.6 ± 1.1	22.1 ± 2.0	22.0 ± 1.9	20.1 ± 1.3	18.6 ± 2.1	19.3 ± 1.7	19.1 ± 1.0	17.3 ± 1.3	17.1± 2.4

Data were taken from pages 108-113 of the study report

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Table 6: Intergroup comparison of food utilization (weight gained (g)/100g food) – carcinogenicity and toxicity studies combined (g) - selected weeks

Week	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
1-4	21.4 ± 0.9	22.2 ± 0.9	21.3 ± 1.1	19.4**± 1.1 (↓9%)	-	14.5 ± 1.2	14.5 ± 1.4	13.4* ± 0.9 (↓8%)	12.7** ± 1.1 (↓12%)	11.6**± 1.1 (↓20%)
5-8	10.2 ± 0.9	9.8 ± 0.8	9.6 ± 0.8	9.7 ± 1.2	10.3± 1.9	5.4 ± 1.3	5.6 ± 0.5	5.4 ± 0.5	5.9 ± 1.1	5.4 ± 1.4
9-13	4.7 ± 0.8	4.8 ± 1.0	4.7 ± 0.7	4.7 ± 0.7	5.6 ± 1.2	3.2 ± 1.0	2.6 ± 0.4	2.6 ± 0.4	2.8 ± 0.4	3.2 ± 0.7
1-13	11.5 ± 0.6	11.8 ± 0.7	11.3 ± 0.4	10.7** ± 0.6 ± (↓7%)	-	7.3 ± 0.6	7.2 ± 0.6	6.7** ± 0.5 (↓8%)	6.7* ± 0.5 (↓8%)	6.4** ± 0.6 (↓12%)

Data were taken from pages 114-115 of the study report

- Food utilization not calculated due to incomplete food consumption data

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Dose rates (based on nominal dietary levels of bicyclopyrone) were calculated in terms of mg bicyclopyrone/kg body weight. Mean values are shown below:

Table 7: Mean dose received (mg/kg bw/day)

	Carcinogenicity study				Chronic toxicity study			
bicyclopyrone (ppm)	5	500	2500	5000	5	500	2500	5000
Males	0.28	28.4	141	280	0.32	32.6	166	335
Females	0.35	35.8	178	368	0.39	41.6	204	404

Data were taken from pages 60-83 of the study report

Water consumption: There were no observable differences between treated and control groups.

Ophthalmoscopic examination: Clinical observations of severe ocular findings were noted throughout treatment from approximately week 4, in which most animals treated at 500 ppm and above were noted with opaque eyes and/or corneal damage (neovascularisation). Additionally, dull corneal surface was seen in 8, 6 and 4% of females at 500, 2500 and 5000 ppm respectively. See table 8.

Table 8: Intergroup comparison of selected organ weights (absolute and covariance analysis) – chronic toxicity study

Organ	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
Eye (s) Opaque (No. Days from – to)	1 (403-737)	3 (387-737)	51 (24-737)	51 (23-737)	51 (23-737)	0	1 (640-710)	52 (24-738)	52 (24-738)	50 (24-738)
Corneal Damage (No. Days from – to)	0	1 (352-359)	51 (24-737)	51 (24-737)	51 (24-737)	0	0	51 (24-738)	52 (24-738)	50 (24-738)

Data were taken from page 43 of the study report

Functional observation battery:

Detailed clinical observations: An absent pupillary reflex was noted in 2/12, 4/12 and 2/12 males and in 4/12, 4/12 and 5/11 females treated at 500, 2500 or 5000 ppm respectively.

Motor activity: There were no treatment-related effects.

Quantitative functional observations: There were no treatment-related effects. Lower hind grip strength was observed in males treated at 2500 and 5000 ppm bicyclopyrone during the functional observational battery assessments. In the absence of any other differences in measured parameters in the functional observational battery or any effect on pathology of the central or peripheral nervous system those isolated findings are considered not to be of toxicological significance.

Haematology: There were no differences in haematological parameters which were considered to be related to treatment across the sampling times.

Blood clinical chemistry: Blood chemistry analysis throughout treatment indicated a consistent increase in both blood glucose and phosphate levels in males and females treated at 2500 ppm and above. A decrease in creatine phosphokinase levels was noted in males and females at 2500 or 5000 ppm at various sampling times throughout the study. In the absence of any clear consistent pattern of change or any evidence of adverse histopathological findings in key organs these changes are considered not to be of toxicological significance.

Coagulation: There were no differences in coagulation parameters which were considered to be related to treatment.

Urinalysis: At termination, an increase in specific gravity coupled with an increase in urinary protein, achieving statistical significance at 500 ppm and above in treated males. Statistically significant increases in urinary ketones at 500 ppm and above were noted in both sexes. Phenylketones are excreted as a consequence of the inhibition of 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) by bicyclopyrone. The increase in urinary ketones in this study is considered to reflect an increase in phenylketones, metabolic products of tyrosine catabolism. Blood pigments were also increased at 2500 and 5000 ppm treated animals.

Sacrifice and pathology:

Organ weights:

Chronic toxicity study: Following adjustment for body weight, a statistical increase in mean covariant kidney weights compared to concurrent control, was noted in males treated at 500, 2500 or 5000 ppm (↑19, 16, 20%). There was no evidence of a dose-response. Mean brain weights (absolute and covariant) were statistically significantly reduced compared to concurrent controls in males treated with 500 ppm and above. There was no evidence of a dose-response. In females, mean heart and uterine weights (absolute and covariant) were statistically significantly decreased at 5000 ppm compared to concurrent control. See table 9.

Table 9: Intergroup comparison of selected organ weights (absolute and covariance analysis) – chronic toxicity study

Organ	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
Kidney (Abs.)	2.60 ± 0.20	2.56 ± 0.21	2.84 ± 0.29	2.82 ± 0.28	2.82 ± 0.24	1.91 ± 0.26	1.89 ± 0.30	1.94 ± 0.23	1.93 ± 0.19	1.77 ± 0.15
Kidney (Adjs.)	2.45 ± 0.05	2.51 ± 0.05	2.91** ± 0.05 (↑19%)	2.83** ± 0.05 (↑16%)	2.93**± 0.05 (↑20%)	1.88 ± 0.06	1.88 ± 0.05	1.91 ± 0.05	1.93 ± 0.05	1.84 ± 0.06

Data were taken from pages 187-192 of the study report

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Carcinogenicity study: Terminal group mean body weights were reduced in comparison to respective controls for both males and females treated with 2500 or 5000 ppm bicyclopyrone. Statistically significantly higher mean covariate liver and kidney weights in males and lower brain and heart weights in both sexes, compared to concurrent controls, were noted and are detailed below. There was no clear evidence of a dose-response. Following adjustment for body weight, a statistically significant ($p<0.01$) increase in mean covariate kidney weight was seen in males treated with 500, 2500 and 5000 ppm. Mean covariant liver weights were statistically significantly increased ($p<0.01$) in males treated at 500, 2500 and 5000 ppm. Mean covariant brain weights were statistically significantly reduced ($p<0.01$) in males at 500 and 5000 ppm and in females at 500, 2500 and 5000 ppm ($p<0.01$). Mean covariant heart weights were statistically significantly decreased in males at 2500 or 5000 ppm ($p<0.05$) and females at 5000 ppm ($p<0.01$). See tables 10 and 11.

Table 10: Intergroup comparison of selected absolute organ weights – carcinogenicity study

Organ	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
Kidney	3.02 ± 0.31	3.27 ± 0.78	3.40** ± 0.58 (↑13%)	3.30 ± 0.44	3.24 ± 0.37	2.33 ± 0.31	2.45 ± 0.32	2.39 ± 0.27	2.27 ± 0.28	2.14* ± 0.27 (↓8%)
Heart	1.50 ± 0.29	1.51 ± 0.17	1.42 ± 0.17	1.35** ± 0.16 (↓10%)	1.33** ± 0.16 (↓11%)	1.13 ± 0.14	1.15 ± 0.13	1.11 ± 0.13	1.04* ± 0.09 (↓8%)	0.98** ± 0.10 (↓13%)
Liver	16.76 ± 2.53	18.29* ± 2.66	18.37* ± 2.99	17.66 ± 2.43	17.40 ± 2.49	12.19 ± 2.29	12.59 ± 2.49	12.29 ± 1.80	11.80 ± 2.10	10.90* ± 1.70

		(↑9%)	(↑10%)							(↓11%)
Brain	2.26 ± 0.10	2.18 ± 0.09	2.10* ± 0.08 (↓7%)	2.11* ± 0.08 (↓7%)	2.08** ± 0.09 (↓8%)	2.02 ± 0.07	2.02 ± 0.06	1.95** ± 0.07 (↓3%)	1.95** ± 0.07 (↓3%)	1.92** ± 0.08 (↓5%)

Data were taken from pages 193-198 of the study report

* Statistically significant difference from control group mean, p<0.05

** Statistically significant difference from control group mean, p<0.01

Table 11: Intergroup comparison of selected organ weights (covariant analysis) – carcinogenicity study

Organ	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
Kidney	2.95 ± 0.07	3.12 ± 0.07	3.40** ± 0.07 (↑15%)	3.39** ± 0.07 (↑15%)	3.53** ± 0.07 (↑20%)	2.24 ± 0.04	2.39* ± 0.04 (↑7%)	2.32 ± 0.04	2.32 ± 0.04	2.31 ± 0.04
Heart	1.48 ± 0.03	1.46 ± 0.03	1.42 ± 0.03	1.38* ± 0.03 (↓7%)	1.36* ± 0.03 (↓8%)	1.11 ± 0.02	1.13 ± 0.02	1.09 ± 0.02	1.06 ± 0.02	1.04* ± 0.02 (↓6%)
Liver	16.34 ± 0.32	17.29 ± 0.32	18.41** ± 0.31 (↑13%)	18.28** ± 0.30 (↑12%)	18.12** ± 0.31 (↑11%)	11.53 ± 0.27	12.09 ± 0.26	11.78 ± 0.26	12.19 ± 0.27	12.22 ± 0.28
Brain	2.15 ± 0.01	2.17 ± 0.01	2.10* ± 0.01 (↓2%)	2.11 ± 0.01	2.09*± 0.01 (↓3%)	2.01 ± 0.01	2.02 ± 0.01	1.94** ± 0.01 (↓3%)	1.95** ± 0.01 (↓3%)	1.93** ± 0.01 (↓4%)

Data were taken from pages 193-198 of the study report

* Statistically significant difference from control group mean, p<0.05

** Statistically significant difference from control group mean, p<0.01

Macroscopic findings: Opaque eyes were recorded in a large number of 500, 2500, and 5000 ppm animals from both the 52 and 104 week studies (see table 8 above). In addition, abnormal shape of the eyes (due to corneal damage) was recorded in 500, 2500 and 5000 ppm animals at 104 weeks. All other necropsy findings were considered background findings associated with this age and strain of rat, on a 52 week toxicity or 104 week carcinogenicity study.

Microscopic findings

Non-neoplastic:

Toxicity study: Minimal to marked keratitis and regenerative hyperplasia of the cornea of the eye were present in all but a few animals given bicyclopyrone at 500 ppm and above (≥83% for keratitis and ≥58% for the regenerative hyperplasia of the cornea). The findings correlated with necropsy findings of opaque eyes and ophthalmology findings of corneal damage. The severity of this lesion was greater in males than in females. The increase in the incidences of keratitis ($p<0.001$) and regenerative hyperplasia of the corneal epithelium ($p<0.001$ for 500 and 5000 ppm and $p<0.01$ for 2500 ppm) were statistically significant when compared to controls.

There was a higher incidence of hypertrophy of follicular cells of the thyroid gland in animals treated at 500 ppm and above (≥66% for males and ≥42% for females). The increase in follicular cell hypertrophy of the thyroid was statistically significant in males treated at 500, 2500 ppm ($p<0.001$) and 5000 ppm ($p<0.01$), and in females treated at 2500 and 5000 ppm ($p<0.05$). There was no clear dose response.

A higher recorded incidence of Harderian gland alteration of the exorbital lachrymal gland reached statistical significance in males treated at 500 ppm ($p<0.01$) and at 5000 ppm ($p<0.05$). Harderian gland alteration in male animals was also associated with minimal to moderate chronic inflammation in the affected acini of the lachrymal gland, and correlated with the necropsy findings of speckled, pale or pale focus recorded in male animals. See table 12.

Table 12: Intergroup comparison of the incidence of selected non-neoplastic microscopic findings – toxicity study (n=12 per sex/per dose)

Finding	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
Eye: Keratitis	0	0	12*** (100%)	10*** (83%)	12*** (100%)	0	0	12*** (100%)	11*** (92%)	11*** (92%)
Eye: Regenerative hyperplasia, corneal	0	0	12*** (100%)	7** (58%)	12*** (100%)	0	0	11*** (92%)	7** (58%)	9*** (75%)
Thyroid: focal cell hypertrophy	0	0	9*** (75%)	10*** (83%)	8*** (66%)	0	0	0	5* (42%)	6* (52%)
Thyroid: focal follicular cell hyperplasia	4	2	2	3	4	1	1	0	4	0

Data were taken from pages 231-263 of the study report
* Statistically significant difference from control group mean, $p<0.05$
** Statistically significant difference from control group mean, $p<0.01$
*** Statistically significant difference from control group mean, $p<0.001$

Carcinogenicity study: Statistically significant increases in the incidence of keratitis (≥73% for both sexes) and regenerative hyperplasia (≥35% for both sexes) in the eye were present in all groups treated at 500 ppm and above ($p<0.001$). There was a statistically significant increase in the incidence of focal follicular cell hyperplasia in the thyroid gland in male animals treated at 5 ($p<0.05$), 500, 2500 ($p<0.01$) and 5000 ppm ($p<0.001$). Incidences were for 5, 500, 2500 and 5000 ppm were 19, 23, 23 and 33% compared to 4% in the control males.

The incidence of focal follicular cell hyperplasia was low (4%) in the control males in this study when compared to the historical control range. In a concurrent study, and one conducted shortly after (see Table 13), the incidence of thyroid hyperplasia in control male rats was 14 and 10%, respectively. Changes observed in the thyroid gland were consistent with a mild perturbation of thyroid function. At dietary concentrations of 500 ppm and above there was a consistent effect on the thyroid gland in male rats. A clear, but not dose related, increase in the incidence of focal cell hypertrophy was noted after 1 year of treatment. After 2 years a clear, but not dose related, increased incidence of focal follicular cell hyperplasia was evident. In contrast, in 5 ppm males, the incidence of focal follicular cell hyperplasia was highlighted as statistically significantly increased when compared to a low concurrent control value after 2 years of treatment whereas there was no evidence of an effect in 5 ppm males in the toxicity phase of the study.

It is concluded that although a treatment related effect on the thyroid at 5 ppm cannot be excluded it is not consistent with the effects noted at 500 ppm and above in that no change in

pathology was apparent after 1 year of treatment. A firm conclusion on the relationship to treatment is further complicated by a low concurrent control incidence focal follicular cell hyperplasia in the carcinogenicity phase.
There was a statistically significant increase in the incidence of acinar cell atrophy in the pancreas of male animals treated at 2500 ppm (50%, $p<0.05$) and 5000 ppm (58%, $p<0.01$).

There was a statistically significant increase in the incidence of Harderian gland alteration in males treated at 5000 ppm (63%, $p<0.05$), and inflammation/inflammatory cell infiltration in the lachrymal glands of male animals treated at 2500 (29%, $p<0.01$) and 5000 ppm (63%, $p<0.001$).

There was a statistically significant increase in the incidence of chronic progressive nephropathy of the kidneys in male animals treated at 5 (63%, $p<0.01$), 500, 2500 and 5000 ppm ($\geq$69%, $p<0.001$), while the incidence of pelvic mineralisation in the kidneys was decreased in males given 5000 ppm ($p<0.05$) and females treated at 500 ppm and above ($p<0.001$ for all 3 groups). An increase in the incidence of pelvic mineralization of the kidneys in females treated at 5 ppm ($p<0.05$) was considered incidental to exposure to bicyclopyrone.

According to the study authors, chronic progressive nephropathy is a common spontaneous age-related finding particularly in male rats. The incidence recorded in males at 5 ppm in this study was within historical control incidence at this laboratory. In addition no effect on kidney weight or urine clinical chemistry analysis was noted at 5 ppm whereas at 500 ppm and above there was a statistically significant increase in group mean covariate kidney weight compared to the concurrent control and a significant increase in urine specific gravity and urinary protein. Historical control data are presented on table 14.

There was a statistically significant decrease in the incidence of cardiomyopathy in male animals treated at 500 (31%, $p<0.05$), 2500 and 5000 ppm (19 and 15%, $p<0.001$).

Table 13: Intergroup comparison of the incidence of selected non-neoplastic microscopic findings – carcinogenicity study (n=52 per sex/dose)

Finding	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
Eye: Keratitis	1	1	46*** (88%)	43*** (83%)	46*** (88%)	0	0	45*** (87%)	45*** (87%)	38*** (73%)
Eye: Regenerative hyperplasia, corneal	1	0	32*** (62%)	33*** (63%)	37*** (71%)	0	0	22*** (42%)	30*** (58%)	18*** (35%)
Thyroid: focal cell hypertrophy	0	1	2	1	1	0	0	1	2	2
Thyroid: focal follicular cell hyperplasia	2	10* (19%)	12** (23%)	12** (23%)	17*** (33%)	3	3	10* (19%)	6	2
Pancreas: acinar atrophy +/- inflammatory cell infiltration	14	9	19	26* (50%)	30** (58%)	7	6	6	6	10
Lachrymal gland: Harderian gland alteration	23	22	31	28	33* (63%)	3	4	3	6	7
Lachrymal gland: Inflammation / inflammatory cell infiltration	4	8	12	15** (29%)	21*** (63%)	1	3	1	1	1
Kidney: Chronic progressive nephropathy	17	33** (63%)	39*** (75%)	40*** (77%)	36*** (69%)	11	12	16	11	11
Kidney: Pelvic mineralisation	8	13	2	3	1* (2%)	29	42* (81%)	10*** (19%)	5*** (10%)	2*** (4%)
Heart: Cardiomyopathy	27	17	16* (31%)	10*** (19%)	8*** (15%)	7	6	6	2	3

Data were taken from pages 264-301 of the study report
* Statistically significant difference from control group mean, $p<0.05$
** Statistically significant difference from control group mean, $p<0.01$
*** Statistically significant difference from control group mean, $p<0.001$

Table 14: **Historical control incidence of selected non-neoplastic microscopic findings in male Crl:Han Wistar rats**

Year of study	2004	2005	2006	2007	2006	2007	2007	2009	Range %
Total number of animals examined	50	100	100	47	108	52	52	52	
Focal follicular cell hyperplasia	0	9	12	1	10	2	7	5	
% incidence	0	9	12	2	10	4	14	10	0-14
Chronic progressive nephropathy	28	28	59	22	30	17	33	17	
% incidence	56	28	59	47	28	33	63	33	28-63

Neoplastic: Squamous cell carcinoma of the cornea of the eye was recorded in 2/52 males animals from each of the groups treated at 500, 2500 and 5000 ppm (all 4%). Squamous cell papilloma of the cornea of the eye was recorded in 1/52 male animals from each of the groups

treated at 500 and 2500 ppm and in 3/52 animals treated at 5000 ppm (2, 2 and 6%). The tumors were associated with keratitis and regenerative hyperplasia of the cornea. According to the study authors, the incidence of the tumors did not reach statistical significance when analysed with the Fisher's Exact Test. See table 15.

Table 15: Intergroup comparison of the incidence of selected neoplastic microscopic findings – carcinogenicity study

Finding	Dietary Concentration of bicyclopyrone (ppm)									
	Males					Females				
	0	5	500	2500	5000	0	5	500	2500	5000
Cornea: Squamous cell carcinoma (malignant)	0	0	2 (4%)	2 (4%)	2 (4%)	0	0	0	0	0
Cornea: Squamous cell papilloma (benign)	0	0	1 (2%)	1 (2%)	3 (6%)	0	0	0	0	0

Data were taken from pages 342-664 of the study report

INVESTIGATOR'S CONCLUSIONS

Dietary administration of bicyclopyrone to rats at 0, 5, 500, 2500 and 5000 ppm, for a period of up to 104 Weeks, was associated with in-life effects (reduced body weights, food consumption and food utilisation) in rats treated at 2500 or 5000 ppm. No evidence of any differences in the survival patterns were seen for the treated groups in either sex when compared to the control animals.

Dietary treatment with bicyclopyrone was associated with the occurrence of squamous cell carcinoma and papilloma, and opacity, keratitis and regenerative hyperplasia of the cornea of the eye at dietary concentrations of 500 ppm and above.

Treatment with bicyclopyrone was associated with non neoplastic findings in the thyroid gland, kidneys (with organ weight increases and urine clinical chemistry changes), exocrine pancreas, heart (with associated organ weight differences) and the lachrymal gland.

The NOEL for findings in the exocrine pancreas, the heart and the lachrymal gland was 500 ppm, while the NOAEL for chronic progressive nephropathy in the kidney and focal follicular cell hyperplasia of the thyroid gland in males was considered to be 5 ppm.

Therefore, a clear No Observed Adverse Effect Level (NOAEL) for this study was considered to be 5 ppm for both males and females equating to 0.28 mg/kg/day NOA449280 in males and 0.35 mg/kg/day in females.

REVIEWER COMMENTS

The purpose of this study was to determine the toxicities in rats resulting from 1-2 years of exposure to bicyclopyrone through the oral route. Five groups of 52 male and 52 female Han Wistar rats were assigned to the Carcinogenicity study and dosed with diets containing 0, 5, 500, 2500 or 5000 ppm bicyclopyrone for at least 104 consecutive weeks. In addition, a chronic toxicity study comprising a further 5 groups of 12 males and 12 females was included and dosed in an identical fashion for a period of 52 consecutive weeks. The equivalent doses for the

carcinogenicity phase of the study were 0, 0.28/0.35, 28.4/35.8, 141/178 and 280/368 mg/kg/day (M/F). The equivalent doses for the chronic toxicity phase of the study were 0, 0.32/0.39, 32.6/41.6, 166/204 and 335/404 mg/kg/day (M/F). Under the conditions of the study, the adverse effects of this study are as follow:
At 5 ppm bicyclopyrone, there was a 2-6% increase in the incidence of opaque eyes and corneal damage in both sexes compared to the control group (0-2%). At 104 weeks in males, there was an increased incidence of thyroid follicular hyperplasia in males (19%) compared to the control group (4%). There was also an increase in the incidence of chronic progressive nephropathy in the kidneys of males (63%) compared to the control group (33%).

At 500 ppm bicyclopyrone, there was a significant increase in the incidence of opaque eyes and corneal damage in both sexes (98-100%) compared to controls (0-2%). There was an increase in the incidence of eye keratitis (88-100% for males and 87-100% for females) and the regenerative corneal hyperplasia (88-100% for males and 42-92% for females) from 52 weeks to 104 weeks compared to the control group (2%). In males, there was an increased incidence of thyroid follicular hypertrophy (75%) at 52 weeks compared to the control group (0%). At 104 weeks in males, there was an increased incidence of thyroid follicular hyperplasia (23%) compared to the control group (19%). This effect occurred in females as well but there was no dose response. There was also an increase in the incidence of chronic progressive nephropathy in the kidneys of males (75%) compared to the control group (33%). In males, there was an increased incidence of squamous cell carcinoma and papilloma (4% and 2%) compared to the control group (0%).

At 2500 ppm bicyclopyrone, there was a significant increase in the incidence of opaque eyes and corneal damage in both sexes compared to controls (98-100%) compared to the control group (0-2%). Decreases in absolute body weights for females were transiently statistically significant through the study (↓5-10%). Relative to the control group, there was a minor decrease in the absolute brain weights of males and females (↓3-7%), and heart weights of females (↓7%). There was an increase in the incidence of eye keratitis (83% for males and 87-92% for females) and regenerative corneal hyperplasia (58-63% for males and 58% for females) from 52 weeks to 104 weeks compared to the control group (2%). In males, there was an increased incidence of thyroid follicular hypertrophy (83%) at 52 weeks compared to the control group (0%). At 104 weeks in males, there was an increased incidence of thyroid follicular hyperplasia (23%) compared to the control group (4%). There was also an increase in the incidence of chronic progressive nephropathy in the kidneys of males (77%) compared to the control group (33%). There was a statistically significant increase in the incidence of acinar cell atrophy in the pancreas of male animals (50%) compared to the control group (27%). In males, there was an increased incidence of squamous cell carcinoma and papilloma (4% and 2%) compared to the control group (0%).

At 5000 ppm bicyclopyrone, there was a significant increase in the incidence of opaque eyes and corneal damage in both sexes (98-100%) compared to the control group (0-2%). Relative to the control group, in both sexes there were significantly lower body weights (↓5-16% for males and ↓5-20% for females). There were also minor changes in food consumption and utilization. There was an increase in the incidence of eye keratitis (88-100% for males and 73-92% for females) and the regenerative corneal hyperplasia (71-100% for males and 35-75% for females) from 52 weeks to 104 weeks compared to the control group (2%). In males, there was an increased incidence of thyroid follicular hypertrophy (66%) at 52 weeks compared to the control group (0%). At 104 weeks in males, there was an increased

incidence of thyroid follicular hyperplasia (33%) compared to the control group (4%). There was also an increase in the incidence of chronic progressive nephropathy in the kidneys of males (69%) at 104 weeks compared to the control group (33%). There was a statistically significant increase in the incidence of acinar cell atrophy in the pancreas of male animals (58%) compared to the control group (27%). In males, there was an increased incidence of squamous cell carcinoma and papilloma (4% and 6%) compared to the control group (0%).

Based upon the effects in this study, the LOAEL for systemic toxicity is 5 ppm (0.28/0.35 mg/kg/day [M/F]) based on a dose dependent increase in the incidence of opaque eyes and corneal damage in both sexes compared to controls, an increased incidence of thyroid follicular hyperplasia in males, and an increased incidence of chronic progressive nephropathy in the kidneys of males. The NOAEL was not established.

The corneal tumors seen in males rats are associated with and likely attributable to significant damage to and regenerative hyperplasia of the cornea seen during the course of the carcinogenicity study with bicyclopyrone at concentrations of 500 ppm and above. The identified mode of action of HPPD inhibiting herbicides results in significantly elevated plasma tyrosine in rats, particularly males. EPA's Cancer Assessment Review Committee determined that in male rats, there was a dose-dependent increase in corneal tumors which were considered treatment related (Rowland et al, September 10, 2014, TXR #0057011). The doses tested were considered to be adequate and not excessive, for assessing carcinogenicity in both sexes. This was based upon increases in corneal opacity, decreased absolute body weights in both sexes at the high dose, and an increased incidence of regenerative corneal hyperplasia in both sexes.

This study is classified as totally reliable **(acceptable/guideline)** as a combined chronic/carcinogenicity study in rats (OPPTS 870.4300; OECD 451). EPA, PMRA (Canada), and APVMA/OCS (Australia) agree regarding the classification of this study but not the regulatory decision. APVMA believes that the chronic progressive nephropathy and thyroid follicular hyperplasia are both within the historical control range and are thus not adverse. It is their conclusion that the NOAEL should be 5 ppm, as all three effects relied on for LOAEL selection are not considered toxicologically adverse. APVMA further disagrees with EPA in thinking that there are no lesions within the study worthy of being considered carcinogenic.

(Robertson B and Perry C, 2012)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anw Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) Date: 03/19/15
EPA Reviewer: Greg Akerman, Ph.D. **Signature:**
Risk Assessment Branch I, Health Effects Division (7509P) Date: 3/18/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Reverse Mutation Test Using Bacteria. OECD 471 (1997): OPPTS 870.5100 (1998): 2000/32/EEC B.13/B.14 (2000)

TEST MATERIAL (PURITY): NOA449280 (94.5%)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Callander R D, (2007). NOA449280 - Bacterial Mutation Assay In S.typhimurium And E.coli. Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK. Laboratory report no. YV7368-REG. 6 February 2007. Unpublished. (Syngenta File No. NOA449280/0038) MRID 47841979

SPONSOR: Syngenta Limited Alderley Park, Macclesfield Cheshire, SK10 4TJ, UK

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a bacterial mutation assay (MRID #47841979), bicyclopyrone (NOA449280) was evaluated over a range of concentrations using strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and two strains of *Escherichia coli* (WP2 (pKM101) and WP2*uvrA* (pKM101)) in the presence and absence of a rat liver - derived metabolic activation system (S9 -mix).

In two separate assays, the test substance did not induce any significant, reproducible increases in the observed number of revertant colonies in any of the tester strains used in the presence of S9 -mix, or in strains TA1537, TA98, TA100, WP2 (pKM101) and WP2*uvrA* (pKM101) in the absence of S9 -mix.

Although bicyclopyrone induced increases in the observed numbers of reverant colonies in strain TA1535 in the absence of S9 -mix that slightly exceeded 2x the concurrent solvent control in two of the three experiments conducted, these increases were not dose-related and were not consistently reproducible. The observed effects are therefore not considered to be due to compound induced mutation.

The sensitivity of the test system, and the metabolic activity of the S9 -mix, were clearly demonstrated by the increases in the numbers of revertant colonies induced by positive control substances.

Bicyclopyrone was non-mutagenic in *S.typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E.coli* strains WP2 (pKM101) and WP2*urvA*(pKM101) in both the presence and absence of S9 -mix.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirement of Test Guideline OPPTS 870.5100; OECD 471 for a bacterial mutation assay.

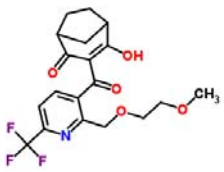
COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown beige powder
Lot/Batch number:	SEZ3AP006
Purity:	94.5%
CAS#:	352010-68-5
Stability of test compound:	Stable

Structure:



Control Materials:

Negative: Not applicable

Solvent control (final concentration): DMSO

Positive control: Nonactivation:

Sodium azide 2.0 µg/plate TA100, TA1535
Daunorubicin hydrochloride 1.0 µg/plate TA98
Acridine mutagen ICR191 2.0 µg/plate TA1537
Mitomycin C 1.0 µg/plate WP2 (pKM101)
N-Ethyl-N´-nitro-N-nitrosoguanidine 1.0 µg/plate WP2 *uvrA* (pKM101)

Activation:

2-Aminoanthracene
1.0 µg/plate TA100, TA98
2.0 µg/plate TA1535, TA1537
20 µg/plate WP2 (pKM101)
Benzo (a)pyrene 5.0 µg/plate WP2 *uvrA* (pKM101)

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

S9 was purchased from MolTox Inc., and was prepared from male Sprague Dawley rats, dosed once daily (by oral gavage) for 3 days with a combined phenobarbital (80 mg/kg bodyweight) and β-naphthoflavone (100 mg/kg) corn oil solution. The treated animals were sacrificed on the day following the third dose. A 25% w/v homogenate (the S9 fraction) was prepared.

The cofactor solution was prepared as a single stock solution of Na2HPO4 (150 mM), KCl (49.5 mM), glucose-6-phosphate (7.5 mM), NADP (Na salt) (6 mM) and MgCl2 (12 mM) in sterile deionised water, and adjusted to a final pH of 7.4.

Test organisms:

***S. typhimurium* strains**

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

***E. coli* strains**

X	WP2 (pKM101)	X	WP2 *uvrA* (pKM101)						

Properly maintained?	X	Yes		No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?	X	Yes		No

Test compound concentrations used
Nonactivated conditions: 5000, 2500, 1000, 500, 200 and 100 µg/plate
Activated conditions: 5000, 2500, 1000, 500, 200 and 100 µg/plate
For all strains triplicate plates were used for all test substance and positive control treatments. For solvent controls 5 plates were used.

Study Design and Methods:
Study dates: Start: 12 May 2006 End: 14 July 2006.

TEST PERFORMANCE

Preliminary Cytotoxicity Assay: Not performed.
Type of Bacterial assay:
- X standard plate test (both experiments –S9, initial experiment +S9)
- X pre-incubation (60 minutes) (second experiment +S9)

Protocol:

Bacterial cultures were prepared from frozen stocks by incubating for 10-12 hours at 37 °C.

The following materials were mixed in a test tube and poured onto the selective agar plates:
- 100 µl Test solution at each dose level, solvent and positive controls;
- 500 µl S9 mix or phosphate buffer;
- 100 µl Bacteria suspension;
- 2 ml Overlay agar containing 50 µM histidine or tryptophan as appropriate.

In the pre-incubation assay 100 µl test solution, 500 µl S9 mix and 100 µl bacterial suspension were mixed in a test tube and incubated at 37°C for 60 minutes. After pre-incubation 2.0 ml overlay agar was added to each tube. The mixture was poured on Vogel Bonner plates.
After the agar was set the plates were incubated upside down for 3 days at 37° C in the dark. For each strain and dose level, three plates were used. For DMSO solvent control, 5 plates were used.
Following incubation all plates were counted using an automated colony counter (Sorcerer® automated counter linked to the Ames Study Manager system [Perceptive Instruments]) adjusted appropriately to permit the optimal counting of mutant colonies.

Statistical analysis: None – see Evaluation Criteria below. According to the OECD guideline 471, a statistical analysis of the data is not mandatory.

Evaluation criteria: A positive response in a (valid) individual experiment is achieved when one or both of the following criteria are met:
- a significant, dose-related increase in the mean number of revertants is observed;
- a two-fold or greater increase in the mean number of revertant colonies (over that observed for the concurrent solvent control plates) is observed at one or more concentrations

A negative result in a (valid) individual experiment is achieved when:

- there is no significant dose-related increase in the mean number of revertant colonies per plate observed for the test substance; and
- in the absence of any such dose response, no increase in colony numbers is observed (at any test concentration) which exceeds 2x the concurrent solvent control.

For a positive response in an individual experiment to be considered indicative of an unequivocal positive, i.e. mutagenic, result for that strain/S9 combination, then the observed effect(s) must be consistently reproducible.

REPORTED RESULTS

Preliminary cytotoxicity assay: Not performed.

Mutagenicity assay:
Mutagenicity data are presented in tables 1-5 below. In two separate assays, the test substance did not induce any significant, reproducible increases in the observed number of revertant colonies in any of the tester strains used in the presence of S9-mix, or in strains TA1537, TA98, TA100, WP2 (pKM101) and WP2 *uvrA* (pKM101) in the absence of S9-mix.

Although bicyclopyrone induced increases in the observed numbers of relevant colonies in strain TA1535 in the absence of S9-mix that slightly exceeded 2x the concurrent solvent control in two of the three experiments conducted, these increases were not dose-related and were not consistently reproducible. The observed effects are therefore not considered to be due to compound-induced mutation.

The positive controls for each experiment induced the expected responses, indicating the strains were responding satisfactorily in each case.

TABLE 1 Data For Experimental Phase 1 (Plate Incorporation: -S9)

Experiment: YV7368 Phase 1

Date Plated: 05/06/2006
Date Counted: 08/06/2006

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	**Y11701/012**	5000 µg	*83.3*	*13.7*	*0.9*	98, 81, 71
		2500 µg	*98.0*	*8.2*	*1.1*	89, 105, 100
		1000 µg	*98.3*	*4.5*	*1.1*	98, 94, 103
		500 µg	*97.7*	*17.6*	*1.1*	79, 100, 114
		200 µg	*82.3*	*2.1*	*0.9*	80, 84, 83
		100 µg	*92.7*	*14.8*	*1.0*	89, 109, 80
	DMSO		*89.6*	*9.5*		76, 84, 94, 94, 100
TA1535	**Y11701/012**	5000 µg	*8.3*	*2.5*	*1.3*	8, 6, 11
		2500 µg	*13.7*	*3.5*	*2.2*	10, 17, 14
		1000 µg	*12.3*	*5.1*	*2.0*	11, 18, 8
		500 µg	*10.0*	*4.6*	*1.6*	9, 6, 15
		200 µg	*8.7*	*3.1*	*1.4*	12, 6, 8
		100 µg	*11.0*	*2.6*	*1.8*	9, 14, 10
	DMSO		*6.2*	*3.7*		4, 11, 5, 2, 9
TA98	**Y11701/012**	5000 µg	*25.7*	*6.0*	*1.4*	32, 20, 25
		2500 µg	*22.0*	*6.1*	*1.2*	18, 19, 29
		1000 µg	*24.0*	*6.0*	*1.3*	18, 24, 30
		500 µg	*18.7*	*7.2*	*1.0*	27, 14, 15
		200 µg	*22.0*	*4.4*	*1.2*	17, 24, 25
		100 µg	*32.5*	*7.8*	*1.7*	38, 27, C
	DMSO		*19.0*	*2.9*		20, 19, 23, 18, 15
WP2 (pKM101)	**Y11701/012**	5000 µg	*55.7*	*6.0*	*1.0*	55, 50, 62
		2500 µg	*57.0*	*4.0*	*1.0*	53, 57, 61
		1000 µg	*69.0*	*13.9*	*1.3*	60, 62, 85
		500 µg	*54.7*	*7.5*	*1.0*	55, 47, 62
		200 µg	*72.7*	*12.0*	*1.3*	72, 85, 61
		100 µg	*64.3*	*10.4*	*1.2*	76, 61, 56
	DMSO		*54.8*	*10.1*		38, 53, 60, 60, 63
WP2 uvrA (pKM101)	**Y11701/012**	5000 µg	*141.0*	*28.4*	*0.9*	151, 109, 163
		2500 µg	*179.7*	*19.5*	*1.2*	160, 180, 199
		1000 µg	*152.3*	*27.2*	*1.0*	121, 166, 170
		500 µg	*179.7*	*84.3*	*1.2*	171, 268, 100
		200 µg	*176.3*	*13.3*	*1.2*	184, 161, 184
		100 µg	*173.0*	*10.0*	*1.1*	163, 183, 173
	DMSO		*153.2*	*17.4*		151, 130, 151, 155, 179
TA100	**NaZ**	2 µg	*715.7*	*48.6*	*8.0*	768, 707, 672
TA1535	**NaZ**	2 µg	*695.3*	*55.0*	*112.2*	645, 687, 754
TA98	**DR**	1 µg	*484.0*	*81.4*	*25.5*	578, 436, 438
WP2 (pKM101)	**MMC**	1 µg	*528.0*	*62.6*	*9.6*	559, 569, 456
WP2 uvrA (pKM101)	**ENNG**	1 µg	*734.3*	*68.0*	*4.8*	737, 665, 801

Key to Positive Controls

NaZ	Sodium Azide
DR	Daunorubicin Hydrochloride
MMC	Mitomycin C
ENNG	N-Ethyl-N'-nitro-N-nitrosoguanidine

Key to Plate Postfix Codes

Data were taken from page 19 of the study report

TABLE 1 Data For Experimental Phase 1 (Plate Incorporation: -S9)

Experiment: YV7368:Phase 1Rpt

Date Plated: 09/06/2006
Date Counted: 12/06/2006

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA1537	**Y11701/012**	5000 µg	*13.7*	*0.6*	*1.0*	14, 14, 13
		2500 µg	*13.3*	*0.6*	*0.9*	13, 14, 13
		1000 µg	*18.3*	*7.0*	*1.3*	19, 11, 25
		500 µg	*21.0*	*6.6*	*1.5*	20, 15, 28
		200 µg	*17.0*	*6.0*	*1.2*	11, 17, 23
		100 µg	*16.3*	*3.1*	*1.2*	19, 17, 13
	DMSO		*14.2*	*0.8*		15, 14, 14, 13, 15
TA1537	**ICR**	2 µg	*802.7*	*132.7*	*56.5*	674, 939, 795

Key to Positive Controls

ICR Acridine Mutagen ICR191

Data were taken from page 20 of the study report

TABLE 2 Data For Experimental Phase 1 (Plate Incorporation: +S9)

Experiment: YV7368 Phase 1

Date Plated: 05/06/2006
Date Counted: 08/06/2006

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	**Y11701/012**	5000 µg	*121.3*	*15.6*	*1.2*	138, 119, 107
		2500 µg	*111.0*	*10.5*	*1.1*	112, 100, 121
		1000 µg	*134.3*	*9.3*	*1.3*	145, 128, 130
		500 µg	*135.0*	*16.6*	*1.3*	116, 147, 142
		200 µg	*143.3*	*12.7*	*1.4*	132, 141, 157
		100 µg	*140.0*	*30.8*	*1.4*	163, 152, 105
	DMSO		*103.6*	*9.0*		119, 104, 98, 100, 97
TA1535	**Y11701/012**	5000 µg	*9.0*	*1.7*	*0.9*	10, 10, 7
		2500 µg	*11.7*	*1.5*	*1.2*	12, 10, 13
		1000 µg	*11.0*	*1.0*	*1.1*	12, 10, 11
		500 µg	*13.3*	*2.1*	*1.3*	15, 14, 11
		200 µg	*10.0*	*2.6*	*1.0*	9, 13, 8
		100 µg	*10.0*	*6.6*	*1.0*	9, 17, 4
	DMSO		*10.0*	*3.5*		5, 8, 13, 13, 11
WP2 (pKM101)	**Y11701/012**	5000 µg	*98.3*	*11.0*	*1.0*	87, 99, 109
		2500 µg	*96.7*	*11.0*	*1.0*	84, 103, 103
		1000 µg	*111.7*	*13.1*	*1.1*	98, 113, 124
		500 µg	*111.3*	*5.9*	*1.1*	109, 107, 118
		200 µg	*102.3*	*25.7*	*1.0*	75, 126, 106
		100 µg	*104.3*	*12.5*	*1.1*	113, 90, 110
	DMSO		*99.0*	*3.5*		99, 97, 105, 97, 97
WP2 uvrA (pKM101)	**Y11701/012**	5000 µg	*239.0*	*24.3*	*1.0*	259, 212, 246
		2500 µg	*244.3*	*7.6*	*1.0*	236, 246, 251
		1000 µg	*227.7*	*24.1*	*0.9*	201, 234, 248
		500 µg	*251.7*	*24.1*	*1.0*	272, 225, 258
		200 µg	*235.7*	*7.6*	*1.0*	239, 227, 241
		100 µg	*252.0*	*7.9*	*1.0*	258, 243, 255
	DMSO		*243.0*	*16.1*		225, 265, 253, 240, 232
TA100	**2AA**	1 µg	*859.7*	*69.6*	*8.3*	931, 792, 856
TA1535	**2AA**	2 µg	*405.3*	*32.0*	*40.5*	406, 373, 437
WP2 (pKM101)	**2AA**	20 µg	*325.7*	*32.6*	*3.3*	328, 292, 357
WP2 uvrA (pKM101)	**BP**	5 µg	*1163.3*	*41.9*	*4.8*	1125, 1157, 1208

Key to Positive Controls

2AA 2-Aminoanthracene
BP Benzo[a]pyrene

Key to Plate Postfix Codes

Data were taken from page 21 of the study report

TABLE 2 Data For Experimental Phase 1 (Plate Incorporation: +S9)

Experiment: YV7368:Phase 1Rpt

Date Plated: 09/06/2006
Date Counted: 12/06/2006

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA1537	**Y11701/012**	5000 µg	*21.0*	*5.2*	*1.2*	18, 18, 27
		2500 µg	*20.7*	*5.5*	*1.2*	18, 17, 27
		1000 µg	*20.3*	*1.5*	*1.2*	20, 22, 19
		500 µg	*22.3*	*3.8*	*1.3*	24, 18, 25
		200 µg	*27.3*	*4.5*	*1.6*	23, 27, 32
		100 µg	*16.0*	*3.6*	*0.9*	20, 15, 13
	DMSO		*17.6*	*3.5*		17, 22, 13, 16, 20
TA98	**Y11701/012**	5000 µg	*31.0*	*5.2*	*1.0*	25, 34, 34
		2500 µg	*29.3*	*5.5*	*0.9*	32, 33, 23
		1000 µg	*30.3*	*4.0*	*0.9*	28, 35, 28
		500 µg	*34.7*	*5.1*	*1.1*	36, 29, 39
		200 µg	*32.3*	*10.2*	*1.0*	25, 44, 28
		100 µg	*39.3*	*1.5*	*1.2*	39, 38, 41
	DMSO		*32.6*	*4.4*		38, 27, 32, 36, 30
TA1537	**2AA**	2 µg	*128.3*	*7.1*	*7.0*	131, 117, 122
TA98	**2AA**	1 µg	*825.7*	*74.6*	*25.3*	822, 753, 902

Key to Positive Controls

2AA 2-Aminoanthracene

Data were taken from page 22 of the study report

TABLE 3 Data For Experimental Phase 2 (Plate Incorporation: -S9)

Experiment: YV7368:Phase 2

Date Plated: 19/06/2006
Date Counted: 22/06/2006

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	**Y11701/012**	5000 µg	*88.0*	*14.1*	*1.0*	75, 86, 103
		2500 µg	*100.3*	*17.7*	*1.1*	80, 112, 109
		1000 µg	*96.3*	*13.3*	*1.1*	103, 81, 105
		500 µg	*89.0*	*12.3*	*1.0*	94, 98, 75
		200 µg	*102.3*	*20.1*	*1.1*	121, 105, 81
		100 µg	*90.7*	*11.6*	*1.0*	85, 104, 83
	DMSO		*90.2*	*9.8*		80, 84, 98, 86, 103
TA1535	**Y11701/012**	5000 µg	*4.7*	*3.5*	*0.8*	1, 5, 8
		2500 µg	*8.3*	*0.6*	*1.4*	8, 9, 8
		1000 µg	*8.5*	*0.7*	*1.5*	8, 9, M
		500 µg	*9.0*	*3.6*	*1.6*	6, 13, 8
		200 µg	*8.3*	*3.8*	*1.4*	4, 10, 11
		100 µg	*8.3*	*3.8*	*1.4*	4, 10, 11
	DMSO		*5.8*	*2.3*		9, 3, 7, 5, 5
TA1537	**Y11701/012**	5000 µg	*13.3*	*4.5*	*1.0*	9, 18, 13
		2500 µg	*13.7*	*1.2*	*1.0*	13, 15, 13
		1000 µg	*13.0*	*4.0*	*1.0*	9, 17, 13
		500 µg	*13.0*	*2.6*	*1.0*	10, 15, 14
		200 µg	*10.0*	*4.0*	*0.8*	10, 6, 14
		100 µg	*8.7*	*1.2*	*0.7*	8, 10, 8
	DMSO		*13.2*	*4.9*		19, 15, 15, 6, 11
TA98	**Y11701/012**	5000 µg	*24.7*	*3.1*	*1.1*	24, 22, 28
		2500 µg	*21.7*	*7.6*	*1.0*	30, 15, 20
		1000 µg	*20.3*	*4.0*	*0.9*	24, 16, 21
		500 µg	*23.0*	*7.8*	*1.0*	28, 27, 14
		200 µg	*14.7*	*6.8*	*0.6*	7, 17, 20
		100 µg	*17.7*	*3.1*	*0.8*	15, 17, 21
	DMSO		*22.6*	*6.1*		14, 20, 28, 22, 29
WP2 (pKM101)	**Y11701/012**	5000 µg	*56.0*	*13.1*	*0.9*	71, 50, 47
		2500 µg	*58.0*	*18.7*	*1.0*	38, 75, 61
		1000 µg	*66.0*	*10.5*	*1.1*	76, 67, 55
		500 µg	*58.7*	*4.9*	*1.0*	53, 61, 62
		200 µg	*63.0*	*4.4*	*1.1*	66, 58, 65
		100 µg	*74.7*	*5.1*	*1.2*	76, 69, 79
	DMSO		*60.0*	*9.6*		58, 50, 76, 58, 58
WP2 uvrA (pKM101)	**Y11701/012**	5000 µg	*179.7*	*57.6*	*1.1*	246, 142, 151
		2500 µg	*152.3*	*4.2*	*1.0*	149, 151, 157
		1000 µg	*144.7*	*12.0*	*0.9*	133, 144, 157
		500 µg	*162.7*	*16.3*	*1.0*	166, 145, 177
		200 µg	*148.0*	*13.9*	*0.9*	140, 140, 164
		100 µg	*162.0*	*26.0*	*1.0*	192, 147, 147
	DMSO		*158.2*	*29.4*		137, 141, 207, 165, 141

Key to Plate Postfix Codes

M Plate missing

Data were taken from page 23 of the study report

TABLE 3 Data For Experimental Phase 2 (Plate Incorporation: -S9)

Experiment: YV7368:Phase 2

Date Plated: 19/06/2006
Date Counted: 22/06/2006

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	**NaZ**	2 µg	*934.3*	*28.1*	*10.4*	964, 931, 908
TA1535	**NaZ**	2 µg	*724.7*	*73.2*	*124.9*	677, 688, 809
TA1537	**ICR**	2 µg	*747.7*	*32.1*	*56.6*	711, 761, 771
TA98	**DR**	1 µg	*527.3*	*25.5*	*23.3*	507, 519, 556
WP2 (pKM101)	**MMC**	1 µg	*476.3*	*35.7*	*7.9*	462, 517, 450
WP2 uvrA (pKM101)	**ENNG**	1 µg	*579.0*	*60.9*	*3.7*	648, 533, 556

Key to Positive Controls		Key to Plate Postfix Codes
NaZ	Sodium Azide	
ICR	Acridine Mutagen ICR191	
DR	Daunomycin Hydrochloride	
MMC	Mitomycin C	
ENNG	N-Ethyl-N'-nitro-N-nitrosoguanidine	

Data were taken from page 24 of the study report

TABLE 4 Data For Experimental Phase 2 (Pre-Incubation: +S9)

Experiment: YV7368:Phase 2

Date Plated: 19/06/2006
Date Counted: 22/06/2006

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA100	**Y11701/012**	5000 µg	*114.3*	*11.0*	*0.9*	102, 123, 118
		2500 µg	*114.3*	*9.9*	*0.9*	103, 119, 121
		1000 µg	*123.3*	*12.7*	*1.0*	112, 137, 121
		500 µg	*120.7*	*9.3*	*1.0*	113, 118, 131
		200 µg	*116.7*	*23.2*	*0.9*	132, 90, 128
		100 µg	*127.3*	*12.1*	*1.0*	140, 116, 126
	DMSO		*123.4*	*11.8*		136, 127, 131, 107, 116
TA1535	**Y11701/012**	5000 µg	*9.3*	*4.2*	*0.8*	6, 14, 8
		2500 µg	*11.0*	*3.6*	*1.0*	12, 7, 14
		1000 µg	*13.3*	*0.6*	*1.2*	13, 13, 14
		500 µg	*11.0*	*1.7*	*1.0*	13, 10, 10
		200 µg	*13.0*	*1.0*	*1.2*	13, 12, 14
		100 µg	*13.7*	*2.3*	*1.2*	15, 11, 15
	DMSO		*11.2*	*2.8*		9, 12, 12, 15, 8
TA1537	**Y11701/012**	5000 µg	*12.7*	*3.2*	*0.6*	15, 9, 14
		2500 µg	*18.3*	*1.5*	*0.9*	20, 17, 18
		1000 µg	*21.0*	*1.0*	*1.0*	21, 22, 20
		500 µg	*20.3*	*4.7*	*1.0*	15, 22, 24
		200 µg	*21.0*	*2.6*	*1.0*	18, 23, 22
		100 µg	*18.7*	*4.0*	*0.9*	18, 15, 23
	DMSO		*21.2*	*5.9*		14, 30, 19, 20, 23
WP2 uvrA (pKM101)	**Y11701/012**	5000 µg	*274.3*	*35.3*	*1.1*	315, 251, 257
		2500 µg	*263.0*	*25.7*	*1.0*	283, 272, 234
		1000 µg	*262.0*	*3.6*	*1.0*	258, 265, 263
		500 µg	*247.7*	*50.5*	*1.0*	220, 217, 306
		200 µg	*245.7*	*11.7*	*1.0*	241, 237, 259
		100 µg	*236.3*	*44.4*	*0.9*	243, 277, 189
	DMSO		*251.0*	*16.7*		235, 237, 257, 276, 250
TA100	**2AA**	1 µg	*333.3*	*45.9*	*2.7*	283, 373, 344
TA1535	**2AA**	2 µg	*97.7*	*32.0*	*8.7*	97, 130, 66
TA1537	**2AA**	2 µg	*78.7*	*19.6*	*3.7*	84, 95, 57
WP2 uvrA (pKM101)	**BP**	5 µg	*820.3*	*110.3*	*3.3*	732, 785, 944

Key to Positive Controls		Key to Plate Postfix Codes
2AA	2-Aminoanthracene	
BP	Benzo[a]pyrene	

Data were taken from page 25 of the study report

TABLE 4 Data For Experimental Phase 2 (Pre-Incubation: +S9)

Experiment: YV7368 Phase 2 Rpt 2

Date Plated: 26/06/2006
Date Counted: 29/06/2006

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA98	**Y11701/012**	5000 µg	*27.3*	*8.6*	*0.9*	29 P, 35 P, 18 P
		2500 µg	*26.3*	*2.1*	*0.8*	28, 24, 27
		1000 µg	*33.0*	*3.0*	*1.0*	36, 30, 33
		500 µg	*33.7*	*3.5*	*1.1*	34, 30, 37
		200 µg	*36.7*	*2.5*	*1.2*	39, 34, 37
		100 µg	*31.0*	*2.6*	*1.0*	34, 29, 30
	DMSO		*31.6*	*7.1*		33, 30, 25, 27, 43
TA98	**2AA**	1 µg	*610.0*	*69.6*	*19.3*	690, 563, 577

Key to Positive Controls

2AA 2-Aminoanthracene

Key to Plate Postfix Codes

P Precipitate

Data were taken from page 26 of the study report

TABLE 4 Data For Experimental Phase 2 (Pre-Incubation: +S9)

Experiment: YV7368 Phase 2 Rpt 5

Date Plated: 11/07/2006
Date Counted: 14/07/2006

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
WP2 (pKM101)	**Y11701/012**	5000 µg	*108.3*	*10.8*	*1.5*	96, 116, 113
		2500 µg	*135.0*	*8.5*	*1.9*	134, 144, 127
		1000 µg	*111.7*	*12.4*	*1.6*	104, 126, 105
		500 µg	*112.3*	*15.5*	*1.6*	125, 117, 95
		200 µg	*112.0*	*34.0*	*1.6*	128, 135, 73
		100 µg	*127.7*	*5.5*	*1.8*	133, 122, 128
	DMSO		*70.2*	*3.6*		70, 65, 73, 69, 74
WP2 (pKM101)	**2AA**	20 µg	*400.0*	*18.0*	*5.7*	385, 420, 395

Key to Positive Controls

2AA 2-Aminoanthracene

Data were taken from page 27 of the study report

TABLE 5 Data For Experimental Phase 3 (Plate Incorporation: -S9)

Experiment: YV7368: Phase 3

Date Plated: 23/06/2006
Date Counted: 26/06/2006

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA1535	**Y11701/012**	5000 µg	*4.3*	*1.2*	*0.9*	3, 5, 5
		2500 µg	*7.0*	*3.6*	*1.4*	10, 8, 3
		1000 µg	*12.0*	*3.6*	*2.4*	8, 13, 15
		500 µg	*8.7*	*4.9*	*1.7*	12, 11, 3
		200 µg	*6.3*	*3.2*	*1.3*	4, 10, 5
		100 µg	*7.0*	*2.6*	*1.4*	10, 5, 6
	DMSO		*5.0*	*1.9*		5, 4, 5, 8, 3
TA1535	**NaZ**	2 µg	*723.0*	*41.7*	*144.6*	771, 696, 702

Key to Positive Controls

NaZ Sodium Azide

Data were taken from page 28 of the study report

INVESTIGATOR'S CONCLUSIONS

It is concluded that, under the conditions of this assay, bicyclopyrone gave a negative, i.e. non-mutagenic, response in*S.typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E.coli* strains WP2 (pKM101) and WP2*urvA*(pKM101) in both the presence and absence of S9 -mix, and in strain TA1535 in the presence of S9 -mix.

REVIEWER'S COMMENTS

Bicyclopyrone (NOA449280) was evaluated over a range of concentrations using strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and two strains of *Escherichia coli* (WP2 (pKM101) and WP2*uvrA* (pKM101)) in the presence and absence of a rat liver - derived metabolic activation system (S9 -mix).

Although bicyclopyrone induced increases in the observed numbers of reverant colonies in strain TA1535 in the absence of S9 -mix that slightly exceeded 2x the concurrent solvent control in two of the three experiments conducted, these increases were not dose-related and were not consistently reproducible. The observed effects are therefore not considered to be due to compound induced mutation. The sensitivity of the test system, and the metabolic activity of the S9 -mix, were clearly demonstrated by the increases in the numbers of revertant colonies induced by positive control substances.

Bicyclopyrone was non-mutagenic in *S.typhimurium* strains TA1535, TA1537, TA98 and TA100 and *E.coli* strains WP2 (pKM101) and WP2*urvA*(pKM101) in both the presence and absence of S9 -mix.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirement of Test Guideline OPPTS 870.5100; OECD 471 for a bacterial mutation assay. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Callander R D, 2007)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/18/15
EPA Reviewer: Greg Akerman, Ph.D. **Signature:** Greg Akerman
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/18/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Reverse Mutation Test Using Bacteria. OECD 471 (1997): OPPTS 870.5100 (1998): 2000/32/EEC B.13/B.14 (2000)

TEST MATERIAL (PURITY): Bicyclopyrone (95.9%)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Sokolowski A, (2010). Bicyclopyrone – Salmonella typhimurium and Escherichia coli Reverse Mutation Assay. Harlan, Cytotest Cell ResearchGmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1326800. 23/11/2012. Unpublished. (Syngenta File No. NOA449280_11117) MRID 47841980

SPONSOR: Syngenta Ltd, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a reverse mutation test (MRID #47841980), the potential for bicyclopyrone (NOA449280) to induce point mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, and TA 100, and the Escherichia coli strains WP2 uvrA pKM 101 and WP2 pKM 101 was tested.

No reduced background growth was observed with and without metabolic activation in all strains used in both experiments. No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), were observed with and without metabolic activation in all strains used in both experiments.

No substantial increase in revertant colony numbers of any of the six tester strains was observed following treatment with bicyclopyrone at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance. Positive control chemicals showed appropriate responses in

the relevant strains.

Bicyclopyrone showed no evidence of mutagenicity in the Salmonella typhimurium and Escherichia coli reverse mutation assay.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirement of Test Guideline OPPTS 870.5100; OECD 471 for a bacterial reverse mutations test.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Not reported
Lot/Batch number:	SMU0BP0028
Purity:	95.9%
CAS#:	352010-68-5
Stability of test compound:	Stable

Structure:

Control Materials:	
Negative:	Untreated plate
Solvent control (final concentration):	DMSO (100 µL/plate)
Positive control:	Nonactivation: Sodium azide 10.0 µg/plate TA100, TA1535 4-nitro-o-phenylene-diamine 10 µg/plate for TA 98 and 50 µg/plate for TA 1537 Methylmethanesulfonate 3 µL/plate, WP2 uvrA pKM101 and WP2 pKM101 Activation: 2-Aminoanthracene 2.5 µg/plate (TA 1535, TA 1537, TA 98, TA 100) and 10 µg/plate (WP2 uvrA pKM 101, WP2 pKM 101)

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other

		X	Other β-naphthoflavone		Other		

The S9 fractions are produced by dilution of the liver homogenate with a KCl solution (1+3) followed by centrifugation at 9000 g. Aliquots of the supernatant are frozen and stored in ampoules at -80 °C. Small numbers of the ampoules can be kept at -20 °C for up to one week. The protein concentration in the S9 preparation was 34.3 mg/mL (lot no. R220110) in the pre-experiment/experiment I and 33.1 mg/mL (lot no. 150110) in experiment II.

The amount of S9 supernatant was 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix: 8mM MgCl2, 33 mM KCl, 5 mM, Glucose-6-phosphate, 4 mM NADP in 100 mM sodium-ortho-phosphate-buffer, adjusted to pH 7.4.

Test organisms:

S. typhimurium strains

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

E. coli strains

X	WP2 (pKM101)	X	WP2 uvrA (pKM101)						

Properly maintained?	X	Yes		No
Checked for appropriate genetic markers (rfa mutation, R factor)?	X	Yes		No

Test compound concentrations used

Preliminary cytotoxicity test: 5000, 2500, 1000, 333, 100, 33, 10 and 3 µg/plate

Nonactivated conditions: 5000, 2500, 1000, 333, 100, 33 µg/plate

Activated conditions: 5000, 2500, 1000, 333, 100, 33 µg/plate

For all strains triplicate plates were used for all test substance and positive control treatments.

Study Design and Methods:
Study dates: Start: 14th April 2010 End: 17th May 2010

TEST PERFORMANCE
Preliminary Cytotoxicity Assay:
Eight concentrations were tested for toxicity and mutation induction each with three replicate plates. The experimental conditions in this pre-experiment were the same as described below for the experiment I (plate incorporation test).

Type of Bacterial assay:
X standard plate test (both experiments –S9, initial experiment +S9)

X pre-incubation (60 minutes) (second experiment +S9)

Protocol:

For each strain and dose level including the controls, three plates were used. The following materials were mixed in a test tube and poured onto the selective agar plates:

100 µL Test solution at each dose level, solvent (negative control) or reference mutagen solution (positive control),

500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),

100 µL Bacteria suspension (cf. test system, pre-culture of the strains),

2000 µL Overlay agar

In the pre-incubation assay 100 µL test solution (solvent control), 100 µL reference mutagen solution (positive control), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacteria suspension were mixed in a test tube and incubated at 37 °C for 60 minutes. After preincubation 2.0 mL overlay agar (45 °C) was added to each tube. The mixture was poured on selective agar plates. After solidification the plates were incubated upside down for at least 48 hours at 37°C in the dark.

Statistical analysis: None – see Evaluation Criteria below. According to the OECD guideline 471, a statistical analysis of the data is not mandatory.

Evaluation criteria:

A test item is considered as a mutagen if:

- A biologically relevant increase in the number of revertants exceeding the threshold of twice the colony count of the corresponding solvent control is observed.
- A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration.
- An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.
- A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

REPORTED RESULTS

Preliminary cytotoxicity assay:

The concentration range of the test item was 3 - 5000 µg/plate. Since no toxic effects were observed, 5000 µg/plate was chosen as maximal concentration.

Mutagenicity assay:

Mutagenicity data are presented in tables 1 and 2 below. The assay was performed with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. Due to contamination of the bacteria suspension of strain TA 100 in experiment II,

this part was repeated under identical conditions (reported as part of experiment II). The test item was tested at the following concentrations:

experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

No reduced background growth was observed in both experiments with and without metabolic activation

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), were observed in both experiments.

No precipitation (visible to the unaided eye) of the test item was observed either in the test tubes or on the incubated agar plates.

No substantial increase in revertant colony numbers of any of the six tester strains was observed following treatment with bicyclopyrone at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls. They showed a distinct increase of induced revertant colonies.

TABLE 1 Summary of Results Experiment I

Study Name: 1326800
Experiment: 1326800 VV plate
Assay Conditions:

Study Code: Harlan CCR 1326800
Date Plated: 20/04/2010
Date Counted: 23/04/2010

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)					
			TA 1535	TA 1537	TA 98	TA 100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	DMSO		12 ± 2	7 ± 4	34 ± 3	130 ± 4	198 ± 21	439 ± 24
	Untreated		11 ± 4	6 ± 1	37 ± 10	138 ± 21	219 ± 8	475 ± 10
	Bicyclopyrone	3 µg	10 ± 4	7 ± 1	30 ± 4	129 ± 8	207 ± 11	440 ± 24
		10 µg	12 ± 2	10 ± 3	31 ± 4	122 ± 7	206 ± 10	450 ± 13
		33 µg	14 ± 3	7 ± 1	30 ± 10	124 ± 9	196 ± 10	453 ± 34
		100 µg	13 ± 1	8 ± 2	30 ± 4	132 ± 5	200 ± 28	476 ± 15
		333 µg	11 ± 3	7 ± 1	28 ± 5	113 ± 9	190 ± 9	440 ± 28
		1000 µg	13 ± 1	9 ± 3	32 ± 10	118 ± 3	194 ± 21	426 ± 31
		2500 µg	12 ± 3	9 ± 2	28 ± 10	123 ± 12	195 ± 8	441 ± 29
		5000 µg	10 ± 6	8 ± 5	20 ± 1	128 ± 13	184 ± 7	434 ± 25
	NaN3	10 µg	1785 ± 9			2128 ± 65		
	4-NOPD	10 µg			306 ± 27			
	4-NOPD	50 µg		80 ± 22				
	MMS	3.0 µL					3621 ± 90	3746 ± 111
With Activation	DMSO		23 ± 3	11 ± 1	34 ± 6	137 ± 5	242 ± 6	516 ± 22
	Untreated		23 ± 4	12 ± 5	36 ± 1	147 ± 11	249 ± 12	525 ± 15
	Bicyclopyrone	3 µg	19 ± 4	12 ± 4	36 ± 11	154 ± 6	232 ± 18	499 ± 9
		10 µg	20 ± 4	14 ± 7	38 ± 8	137 ± 3	236 ± 25	488 ± 30
		33 µg	21 ± 7	11 ± 4	36 ± 4	133 ± 5	212 ± 15	481 ± 7
		100 µg	21 ± 2	14 ± 5	39 ± 9	128 ± 5	231 ± 12	475 ± 31
		333 µg	14 ± 1	10 ± 2	44 ± 10	133 ± 10	237 ± 11	510 ± 20
		1000 µg	21 ± 5	13 ± 4	36 ± 3	154 ± 6	235 ± 26	505 ± 24
		2500 µg	17 ± 2	11 ± 5	31 ± 11	140 ± 16	233 ± 10	501 ± 13
		5000 µg	15 ± 8	14 ± 1	33 ± 8	155 ± 12	235 ± 2	480 ± 36
	2-AA	2.5 µg	495 ± 29	581 ± 76	3485 ± 110	3605 ± 6		
	2-AA	10.0 µg					1310 ± 142	1920 ± 85

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Data were taken from page 24 of the study report

TABLE 2 Summary of Results Experiment II

Study Name: 1326800
Experiment: 1326800 HV 2 Pre
Assay Conditions:

Study Code: Harlan CCR 1326800
Date Plated: 04/05/2010 / 11/05/2010*
Date Counted: 07/05/2010 / 17/05/2010*

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)					
			TA 1535	TA 1537	TA 98	TA 100*	WP2 pKM101	WP2 uvrA pKM101
Without Activation	DMSO		15 ± 1	8 ± 1	26 ± 2	124 ± 21	195 ± 16	480 ± 14
	Untreated		15 ± 2	16 ± 4	32 ± 6	152 ± 14	236 ± 18	531 ± 37
	Bicyclopyrone	33 µg	14 ± 1	8 ± 1	26 ± 4	128 ± 7	204 ± 16	462 ± 32
		100 µg	14 ± 2	11 ± 3	30 ± 3	131 ± 2	183 ± 25	488 ± 33
		333 µg	13 ± 4	10 ± 2	24 ± 4	133 ± 10	195 ± 22	466 ± 8
		1000 µg	10 ± 2	8 ± 1	26 ± 10	125 ± 12	208 ± 16	467 ± 21
		2500 µg	9 ± 1	9 ± 5	26 ± 6	93 ± 11	192 ± 23	497 ± 46
		5000 µg	10 ± 3	7 ± 1	20 ± 6	92 ± 2	177 ± 17	437 ± 48
	NaN3	10 µg	1891 ± 67			2116 ± 172		
	4-NOPD	10 µg			413 ± 99			
	4-NOPD	50 µg		81 ± 6				
	MMS	3.0 µL					2406 ± 356	2442 ± 264
With Activation	DMSO		19 ± 4	12 ± 2	35 ± 7	148 ± 4	220 ± 30	509 ± 28
	Untreated		19 ± 4	14 ± 2	36 ± 1	153 ± 15	236 ± 51	553 ± 18
	Bicyclopyrone	33 µg	19 ± 3	10 ± 5	36 ± 2	153 ± 4	234 ± 30	542 ± 5
		100 µg	17 ± 4	10 ± 3	33 ± 2	142 ± 19	259 ± 46	526 ± 144
		333 µg	18 ± 3	10 ± 2	36 ± 2	151 ± 5	220 ± 27	499 ± 15
		1000 µg	20 ± 4	12 ± 4	30 ± 8	135 ± 5	282 ± 81	510 ± 22
		2500 µg	21 ± 1	11 ± 2	30 ± 4	161 ± 24	259 ± 56	556 ± 82
		5000 µg	18 ± 4	8 ± 3	37 ± 6	144 ± 13	239 ± 9	510 ± 22
	2-AA	2.5 µg	466 ± 13	490 ± 19	3161 ± 691	3051 ± 108		
	2-AA	10.0 µg					3453 ± 5	1801 ± 97

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

* = repeated experiment

Data were taken from page 25 of the study report

INVESTIGATOR'S CONCLUSIONS

During the described mutagenicity test and under the experimental conditions reported, bicyclopyrone did not induce point mutations by base pair changes or frameshifts in the genome of the strains used. Bicyclopyrone is considered to be non-mutagenic in the Salmonella typhimurium and Escherichia coli reverse mutation assay.

REVIEWER'S COMMENTS

The potential for bicyclopyrone (NOA449280) to induce point mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, and TA 100, and the Escherichia coli strains WP2 uvrA pKM 101 and WP2 pKM 101 was tested.

Bicyclopyrone showed no evidence of mutagenicity in the Salmonella typhimurium and Escherichia coli reverse mutation assay.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirement of Test Guideline OPPTS 870.5100; OECD 471 for a bacterial reverse mutations

test. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Sokolowski A, 2010)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:**
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/18/15
EPA Reviewer: Greg Akerman, Ph.D. **Signature:**
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/18/2015
TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997): OPPTS 870.5300 (1998): 2000/32/EEC B.17 (2000)

TEST MATERIAL (PURITY): NOA449280 (94.5%)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Booth E, 2006. NOA449280 - L5178Y TK +/- Mouse Lymphoma Mutation Assay. Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK. Laboratory Report No. VV0349-REG. 25-09-2006. Unpublished. (Syngenta File No. NOA449280/0035) MRID 47841981

SPONSOR: Syngenta Limited, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a Mouse Lymphoma Mutation Assay (MRID #47841981), L5178Y TK+/- mouse lymphoma cells were treated *in vitro* with various concentrations of bicyclopyrone (NOA449280), both in the presence and absence of a rat liver derived auxiliary metabolic system (S9-mix). Large and small mutant colonies were scored for all cultures in each experiment. Mutant frequencies were assessed by cell growth in the presence of trifluorothymidine after a 48 hour expression time. Bicyclopyrone was tested both in the presence and absence of S9-mix in two independent experiments.

Bicyclopyrone was tested up to a maximum concentration of 3994 µg/mL in the presence and absence of S9-mix. This concentration is approximately equivalent to 10 mM and as such is the limit concentration for this assay. Minimum survival levels, compared to the solvent control cultures, of 35 % and 34 % were observed in cultures treated with the maximum concentration of bicyclopyrone evaluated for mutant frequency in the presence and absence of S9-mix respectively.

No significant increases in mutant frequency were observed in cultures treated with bicyclopyrone in either the presence or absence of S9-mix in either of the independent experiments. The positive controls induced appropriate increases in mutant frequency in all mutation experiments thus demonstrating the activity of the S9-mix and that the assay was performing satisfactorily in being capable of detecting known mutagens.

Bicyclopyrone showed no evidence of mutagenicity in L5178Y TK+/- cells treated *in vitro* in either the presence or absence of S9-mix.

This study is classified as totally reliable (**acceptable/guideline)** and satisfies the guideline requirement of Test Guideline OPPTS 870.5300; OECD 476 for *in vitro* mammalian cell gene mutation test.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown beige powder.
Lot/Batch number:	SEZ3AP006
Purity:	94.5%
CAS#:	352010-68-5
Stability of test compound:	Not specified

Structure:

Control Materials:

Negative:	None
Solvent control (final concentration):	DMSO 10µl/ml
Positive control:	Absence of S9 mix: Ethylmethanesulphonate, 500 µg/mL Presence of S9 mix: Benzo (a) pyrene, 1 µg/mL

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

The treated animals were sacrificed on the day following the third dose. A 25% w/v homogenate (the S9 fraction) was prepared according to the method given in Callander *et al* (1995).

The co-factor solution was prepared as a stock solution of 75 mM NADP (disodium salt) and 1200mM glucose-6-phosphate (monosodium salt) in RPMI 1640 culture medium with a final pH adjusted to 7.5. Both the S9 fraction and the co-factors were added at 1% (200µl of each added to the 20ml cell culture).

Test cells: mammalian cells in culture

X	Mouse lymphoma L5178Y cells		V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others

Media: RPMI 1640				
Properly maintained?	X	Yes		No
Periodically checked for Mycoplasma contamination?	X	Yes		No
Periodically checked for karyotype stability?		Yes	X	No
Periodically "cleansed" against high spontaneous background?		Yes	X	No

X indicates those that apply

Locus Examined:		**Thymidine kinase (TK)**		**Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**		**Na+/K+ ATPase**
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)		6-thioguanine (6-TG)		
	X	Trifluorothymidine (TFT)				

X indicates those that apply

Test compound concentrations used:

Absence of S9 mix 3994, 2995, 1997, 1497, 998, 499, 250 and 125µg/mL
Presence of S9 mix 3994, 2995, 1997, 1497, 998, 499, 250 and 125µg/mL

Study Design and Methods:

Study dates: Start: 31st May 2006 End: 3rd July 2006

Test performance:

Cell treatment:

Aliquots of the test substance, solvent control or positive controls were administered to duplicate cultures as appropriate to the experimental design. The cultures were treated for 4 hours. During this period the treated cell cultures were rotated on a roller apparatus in a 37°C hot room. At the end of the treatment period the cultures were centrifuged at 250 x g for 5 minutes, the supernatants removed and the cell pellet resuspended in 50 mL of fresh culture medium.

The effect of bicyclopyrone on the pH and osmolality of the treatment medium was investigated as changes in pH and increases in osmolality have been reported to result in artefactual responses in genotoxicity assays. Survival was measured by relative total growth (RTG). RTG is a measure of growth of test cultures both during the two-day expression and cloning phases of the assay, relative to the vehicle control.
The post-treatment cultures were returned to the roller apparatus in the 37°C hot room for a 48 hour expression period. To maintain exponential growth during the expression time, each culture was counted and, where appropriate, diluted daily to give approximately 2 x 10^5 cells per mL in 50 mL, thereby ensuring approximately 10^7 cells at each subculture.

Mutation assay:
After the 48 hour expression period, the mutation assay was performed. The cell density of each culture was determined and the cultures were then divided into two series of dilutions. The first was to form the cultures for assessment of mutants by TFT selection; the second was to assess the viability of the cultures (in the absence of TFT).

For the assessment of mutants, a sample of each of the post-expression cultures was diluted to give 50 mL at 1 x 10^4 cells per mL. TFT was then added to the mutation cultures to give a final concentration of 4 µg/mL. Each TFT treated culture was then dispensed at 200 µL per well into 2 x 96 well microwell plates (2000 cells per well). These plates were then incubated (37°C, 5% CO_2, 98% relative humidity) to allow cell growth.

For the assessment of viability, a sample from each mutation culture (at 1 x 10^4/mL) was diluted to give 50 mL at 8 cells per mL. No TFT was added to these cultures. Each viability culture was then dispensed and incubated as for the mutation cultures.

Data evaluation
Cell growth in individual microwell plates was assessed after 10-13 days using a dissecting microscope. The viability plates were scored for the number of wells containing no cell growth (negative wells). The mutation plates were scored so that each well contained either a small colony (considered to be associated with clastogenic effects), a large colony (considered to be associated with gene mutation effects) or no colony.

Statistical Methods:
The Study Director, in consultation with the Study Statistician, considered that statistical analysis of the data was not necessary.

Evaluation Criteria: Each well of the mutation plates (those containing TFT) was scored as containing either, a small colony, a large colony or no colony according to the following criteria:

Small Colony - a small colony was one whose average diameter was less than 25% of the diameter of the well and was usually around 15% of the diameter of the well. A small colony should also have shown a dense clonal morphology.

Large Colony - a large colony was one whose average diameter was greater than 25% of the diameter of the well. A large colony should also have shown less densely packed cells, especially around the edges of the colony.

Any well which contained more than one small colony was scored as a small colony. Any well which contained more than one large colony was scored as a large colony. Any well which contained a combination of large and small colonies was scored as a large colony.

An empty well was one which contained no cell growth.

RESULTS AND DISCUSSION

Preliminary toxicity assay: Not performed.

Mutation assay:

Results are listed in tables 1 and 2. The maximum concentration of bicyclopyrone considered appropriate for testing in the mutation experiments was determined as 3994 µg/mL in the presence and absence of S9-mix. This concentration is approximately equivalent to 10 mM and as such is the limit concentration for this assay. This concentration resulted in survival levels relative to the solvent control of 47 % and 35 % in the presence of S9-mix in the first and second experiments respectively, and 45 % in the absence of S9-mix in the second experiment. In the first experiment in the absence of S9- mix, the maximum concentration evaluated for mutant frequency was 2995 µg/mL resulting in 34 % survival.

Treatment of the culture medium with concentrations of the test substance used in this study had no significant effect on osmolality or pH.

No significant increases in mutant frequency, compared to the solvent control cultures, were observed in cultures treated with bicyclopyrone at any concentration tested in either the presence or absence of S9-mix.

The positive controls, EMS and BP, induced appropriate increases in mutant frequency in all mutation experiments, demonstrating the activity of the S9-mix and that the assay was performing satisfactorily in being capable of detecting known mutagens.

Table 1: Summary of Data for Experimental Phase 1

Without S9-mix			With S9-mix		
Concentration (µg/ml)	Mean % Relative Total Growth	Mean Mutant Frequency (x 10^{-4})	Concentration (µg/ml)	Mean % Relative Total Growth	Mean Mutant Frequency (x 10^{-4})
NOA449280			NOA449280		
3994	5	b	3994	47	1.0
2995	34	1.2	2995	69	1.1
1997	51	1.1	1997	77	1.1
1497	53	1.0	1497	69	1.1
998	72	0.8	998	70	1.1
499	84	1.3	499	87	0.9
250	105	1.0	250	113	0.9
125	106	1.0	125	117	0.7
SOLVENT CONTROL			SOLVENT CONTROL		
DMSO (10 µl/ml)	100	0.7	DMSO (10 µl/ml)	100	0.8
POSITIVE CONTROL			POSITIVE CONTROL		
EMS 500	61	7.9	BP 1	71	4.6

b = not counted due to excessive toxicity

Data were taken from page 21 of the study report

Table 2: Summary of Data for Experimental Phase 2

Without S9-mix

Concentration (µg/ml)	Mean % Relative Total Growth	Mean Mutant Frequency (x 10^{-6})
NOA449280		
3994	45	1.2
3500	39	1.7
3000	50	1.1
2000	126	1.1
1000	115	1.1
500	137	0.6
SOLVENT CONTROL		
DMSO (10 µl/ml)	100	1.0
POSITIVE CONTROL		
EMS 500	55	7.8

With S9-mix

Concentration (µg/ml)	Mean % Relative Total Growth	Mean Mutant Frequency (x 10^{-6})
NOA449280		
3994	35	1.9
3500	42	1.4
3000	55	1.5
2000	75	0.8
1000	76	0.8
500	97	1.1
SOLVENT CONTROL		
DMSO (10 µl/ml)	100	1.2
POSITIVE CONTROL		
BP 1	24	11.9

Data were taken from page 22 of the study report

INVESTIGATOR'S CONCLUSIONS

It is concluded that, under the conditions of this assay, bicyclopyrone is not mutagenic in L5178Y TK+/- cells treated *in vitro* in either the presence or absence of S9-mix.

REVIEWER'S COMMENTS

In a Mouse Lymphoma Mutation Assay, L5178Y TK+/- mouse lymphoma cells were treated *in vitro* with various concentrations of bicyclopyrone (NOA449280), both in the presence and absence of a rat liver derived auxiliary metabolic system (S9-mix). Large and small mutant colonies were scored for all cultures in each experiment. Mutant frequencies were assessed by cell growth in the presence of trifluorothymidine after a 48 hour expression time. Bicyclopyrone was tested both in the presence and absence of S9-mix in two independent experiments.

Bicyclopyrone showed no evidence of mutagenicity in L5178Y TK+/- cells treated *in vitro* in either the presence or absence of S9-mix.

This study is classified as totally reliable (**acceptable/guideline)** and satisfies the guideline requirement of Test Guideline OPPTS 870.5300; OECD 474 for *in vitro* mammalian cell gene mutation test. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Booth E, 2006)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anw Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) Date: 03/18/15
EPA Reviewer: Greg Akerman, Ph.D. **Signature:** Greg Akerman
Risk Assessment Branch I, Health Effects Division (7509P) Date: 3/18/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: *In Vitro* Mammalian Chromosome Aberration Test. OECD 473 (1997): OPPTS 870.5375 (1998): 2000/32/EC B10 (2000)

TEST MATERIAL (PURITY): NOA449280 (94.5%)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Fox, V (2006). NOA449280 - In Vitro Cytogenetic Assay In Human Lymphocytes. Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield Cheshire, SK10 4TJ, UK. Laboratory Report No. SV1367-REG. 23/10/2006. Unpublished. (Syngenta File No. NOA449280/0036) MRID 47841982

SPONSOR: Syngenta Limited, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In an *in vitro* cytogenetic assay (MRID #47841982), Bicyclopyrone (NOA449280) was evaluated for its clastogenic potential in human lymphocytes in two separate experiments treated in the presence and absence of a rat liver-derived metabolic activation system (S9-mix). Cultures treated with bicyclopyrone were selected for chromosomal aberration analysis along with the appropriate solvent and positive control cultures. There were two experiments -S9 mix; 1250, 750 and 500 µg/mL (experiment 1) and 500, 250 and 125 µg/mL (experiment 2). There were two experiments +S9 mix; 1250, 750 and 500µg/mL (experiment 1), and 1500, 1375 and 1250µg/mL (experiment 2).

The highest concentrations selected for chromosome aberration analysis were based on reductions in mitotic activity. Concentration-related reductions in mitotic activity were observed in cultures from both experiments, thus demonstrating that bicyclopyrone is biologically active in this test system. No statistically or biologically significant increases in the percentage of aberrant cells, compared to the solvent control values, were recorded in cultures from either experiment treated in either the presence or absence of S9-mix.

The sensitivity of the test system, and the metabolic activity of the S9-mix employed, were clearly demonstrated by the increases in the percentage of aberrant cells induced by the positive control agents, mitomycin C and cyclophosphamide.

Bicyclopyrone was negative for clastogenicity in cultured human lymphocytes treated *in vitro* in either the presence or absence of S9-mix.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirement of Test Guideline OPPTS 870.5375; OECD 473 for *in vitro* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown beige powder.
Lot/Batch number:	SEZ3AP006
Purity:	94.5%
CAS#:	352010-68-5
Stability of test compound:	Not specified

Structure:

Control Materials:

Negative:	None
Solvent control (final concentration):	DMSO 10µl/ml
Positive control:	Absence of S9 mix: Mitomycin C, 0.5µg/mL (3 hour treatment), 0.2µg/ml (20 hour treatment) Presence of S9 mix: Cyclophosphamide 50µg/mL

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

The metabolic activation system (S9-mix) used in this study was prepared as required (on each day of culture treatment) as a 1:1 mixture of S9 fraction and cofactor solution.

The cofactor solution was prepared as a single stock solution of Na_2HPO_4 (150mM), KCl (49.5mM), glucose-6-phosphate (7.5mM), NADP (Na salt) (6mM) and $MgCl_2$ (12mM) in sterile double deionised water and adjusted to a final pH of 7.4.

Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
X	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. Equal volumes of blood from 2 donors (female for Experiment 1 and male for Experiment 2) were pooled together for each experiment. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
	Chinese hamster ovary (CHO) cells

Media: RPMI-1640 (Dutch modification)				
Properly maintained?	X	Yes		No
Periodically checked for *Mycoplasma* contamination?		Yes	X	No
Periodically checked for karyotype stability?		Yes	X	No

Test compound concentrations used:

Absence of S9 mix	Experiment 1	1250, 750 and 500 µg/mL
	Experiment 2	500, 250 and 125 µg/mL
Presence of S9 mix	Experiment 1	1250, 750 and 500µg/mL
	Experiment 2	1500, 1375 and 1250µg/mL

Study Design and Methods:
Study dates: Start: 15th May 2006 End: 25th July 2006

TEST PERFORMANCE
Preliminary Cytotoxicity Assay: Not performed.

Cytogenetic Assay:

Cell exposure time:		**Test Material**	**Solvent Control**	**Positive Control**
- S9 mix	Experiment 1	3h	3h	3h
+ S9 mix		3h	3h	3h
-S9 mix	Experiment 2	20h	20h	20h
+ S9 mix		3h	3h	3h

Spindle inhibition:	
Inhibitor used/ concentration:	Colcemid 0.4 µg/mL
Administration time:	2 hours (before cell harvest)

Cell harvest time after termination of treatment:	Test Material	Solvent Control	Positive Control
- S9 mix (3 hour treatment)	17h	17h	17h
+ S9 mix (3 hour treatment)	17h	17h	17h
- S9 mix (20 hour treatment)	0h	0h	0h

Details of slide preparation: Approximately 48 hours after culture establishment, aliquots of the test substance, solvent control or positive controls were administered to duplicate cultures as appropriate to the experiment design. In addition, 200µl of a 1:1 mix of S9 and co-factor solution was added to each culture to be treated in the presence of S9-mix. Cultures from both experiments in the presence of S9-mix and Experiment 1 in the absence of S9-mix were treated for a period of 3 hours at 37°C, after which the culture medium was removed following centrifugation and replaced with fresh supplemented RPMI-1640 culture medium. The cultures were re-incubated at 37°C for the remainder of the 68 hour growth period. Cultures from Experiment 2 in the absence of S9-mix were treated for a period of 20 hours until the end of the 68 hour growth period.

Approximately 2 hours prior to harvesting, the cultures were treated with colcemid at a final concentration of 0.4µg/ml. Sixty-eight hours after culture establishment, the cultures were centrifuged, the supernatant was removed and the cells were re-suspended in approximately 10ml of 0.075M KCl at room temperature for approximately 10 minutes. The cultures were centrifuged, the supernatant was removed and the remaining cells were fixed in freshly prepared methanol/glacial acetic acid fixative (3:1 v/v) added dropwise and made up to a volume of approximately 10ml. The fixative was removed following centrifugation and replaced with freshly prepared fixative. After at least two subsequent changes of fixative, slides were made by dropping the cell suspension on to clean, moist labelled microscope slides. The slides were air dried, stained in filtered Giemsa stain (10% Gurr's R66 in buffered [pH 6.8] double deionised water) for 7 minutes, rinsed in water, air-dried and mounted with coverslips in DPX.

Metaphase analysis

No. of cells examined per dose: 100				
Scored for structural?	X	Yes		No
Scored for numerical?	X	Yes (polyploidy noted if observed)		No
Coded prior to analysis?	X	Yes		No

Evaluation criteria: The percentages of aberrant metaphases were calculated for each treatment scored, both including and excluding cells with only gap-type aberrations.

The Fisher Exact Probability Test (one-sided) was used to evaluate statistically the percentage of metaphases showing aberrations (excluding cells with only gap-type aberrations). Data from each treatment group, in the presence and absence of S9-mix, was compared with the respective solvent control group value. The data have been interpreted as follows:

- No statistically significant increase in the percentage of aberrant cells (at any concentration) above concurrent solvent control values - **NEGATIVE**.

- A statistically significant increase in the percentage of aberrant cells above concurrent solvent control values, which falls within the laboratory solvent control range - **NEGATIVE**.
- An increase in the percentage of aberrant cells, at least at one concentration, which is substantially greater than the laboratory historical solvent control values - **POSITIVE**.
- A statistically significant increase in the percentage of aberrant cells which is above concurrent solvent values and which is above the historical solvent control range upper value but below that described in (c) may require further evaluation.

Statistical analysis: Data were evaluated for statistical significance using the Fisher Exact Probability Test (one-sided).

REPORTED RESULTS

Preliminary cytotoxicity assay: Not performed.

Cytogenetic assay:

Significant reductions in mean mitotic activity, compared to the solvent control values, were observed in cultures from both Experiment 1 (49% +S9-mix; 33% -S9-mix) and Experiment 2 (64% +S9-mix; 60% -S9-mix) treated with the highest concentrations of bicyclopyrone selected for chromosomal aberration analysis (See tables 1 and 2). Cultures treated with higher concentrations of bicyclopyrone (NOA449280) were considered not to be suitable for chromosomal aberration analysis due to lack of metaphases and/or cytotoxic effects on the chromosomes as a result of toxicity.

Treatment of the culture medium with bicyclopyrone up to 1500 µg/mL had no significant effect on osmolality or pH.

No statistically or biologically significant increases in the percentage of aberrant cells, above the solvent control values, were recorded in cultures in either Experiment 1 or Experiment 2 treated with bicyclopyrone in either the presence or absence of S9-mix.

The positive control materials, mitomycin C and cyclophosphamide induced statistically and biologically significant increases in the percentage of aberrant cells, compared to the solvent control cultures.

Table 1: Mean Chromosomal Aberrations and Mitotic Indices in The Absence of Metabolic Activation

Treatment		Mean % Aberrant Cells Excluding Gaps	Mean % Mitotic Index
Experiment 1 – 3 hour treatment			
Solvent Control	10 µL/mL	2.00	5.1
Mitomycin C	0.5 µg/mL	55.00**	2.7Δ
NOA449280			
	1250 µg/mL	2.50	3.4
	750 µg/mL	1.90	3.9
	500 µg/mL	2.50	6.1
Experiment 2 – 20 hour treatment			
Solvent Control	10 µL/mL	4.00	10.9
Mitomycin C	0.2 µg/mL	44.00**	4.4Δ
NOA449280			
	500 µg/mL	3.00	4.4
	250 µg/mL	1.00	6.4
	125 µg/mL	1.00	9.9

** Statistically significant increase in the percentage of aberrant cells at $p<0.01$ using Fisher's Exact Test (one-sided).
Δ Positive control mitotic index and % aberrant cells are determined from a single culture.
Data were taken from page 22 of the study report

Table 2: Mean Chromosomal Aberrations and Mitotic Indices in The Presence of Metabolic Activation

Treatment		Mean % Aberrant Cells Excluding Gaps	Mean % Mitotic Index
Experiment 1 – 3 hour treatment			
Solvent Control	10 µL/mL	0.50	6.1
Cyclophosphamide	50 µg/mL	40.00**	2.8Δ
NOA449280			
	1250 µg/mL	1.50	3.1
	750 µg/mL	0.50	4.1
	500 µg/mL	0.50	5.7
Experiment 2 – 3 hour treatment			
Solvent Control	10 µL/mL	3.50	5.00
Cyclophosphamide	50 µg/mL	66.67**	2.9Δ
NOA449280			
	1500 µg/mL	6.00	1.8
	1375 µg/mL	4.00	2.7
	1250 µg/mL	5.00	3.7

** Statistically significant increase in the percentage of aberrant cells at $p<0.01$ using Fisher's Exact Test (one-sided).
Δ Positive control mitotic index and % aberrant cells are determined from a single culture.
Data were taken from page 23 of the study report

INVESTIGATOR'S CONCLUSIONS

It is concluded that, under the conditions of this assay, bicyclopyrone is not clastogenic to cultured human lymphocytes treated *in vitro* in either the presence or absence of S9-mix.

REVIEWER COMMENTS

In an *in vitro* cytogenetic assay, bicyclopyrone (NOA449280) was evaluated for its clastogenic potential in human lymphocytes in two separate experiments treated in the presence and absence of a rat liver-derived metabolic activation system (S9-mix). Cultures treated with bicyclopyrone were selected for chromosomal aberration analysis along with the appropriate solvent and positive control cultures. There were two experiments -S9 mix; 1250, 750 and 500 µg/mL (experiment 1) and 500, 250 and 125 µg/mL (experiment 2). There were two experiments +S9 mix; 1250, 750 and 500µg/mL (experiment 1), and 1500, 1375 and 1250µg/mL (experiment 2).

Bicyclopyrone was negative for clastogenicity in cultured human lymphocytes treated *in vitro* in either the presence or absence of S9-mix.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirement of Test Guideline OPPTS 870.5375; OECD 474 for *in vitro* cytogenetic mutagenicity data. EPA, PMRA (Canada), and AMPVA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Fox V, 2006)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Ann Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/18/15
EPA Reviewer: Greg Akerman, Ph.D. **Signature:** Greg Akerman
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/18/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Rat bone marrow micronucleus test OECD 474 (1997): 2000/32/EEC B.12 (2000)

TEST MATERIAL (PURITY): NOA449280 (94.5%)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Honarvar N (2008). NOA449280 - Micronucleus Assay in Bone Marrow Cells of the Rat. RCC, Cytotest Cell Research GmbH (RCC-CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1141200. 22/02/2008. Unpublished. (Syngenta File No. NOA449280/0104) MRID 47841984

SPONSOR: Syngenta Ltd, Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, United Kingdom.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a Micronucleus Assay in Bone Marrow Cells of the Rat (MRID #47841984), the potential of bicyclopyrone (NOA449280) to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the rat was investigated. The test substance was formulated in 0.5%, carboxymethyl cellulose (CMC), which was also used as the vehicle control. The volume administered orally was 10 mL/kg bodyweight (b.w.). At 24 h and 48 h after a single administration of the test substance, the bone marrow cells were collected for micronuclei analysis.

Five males per test group were evaluated for the occurrence of micronuclei. At least 2000 polychromatic erythrocytes (PCEs) per animal were scored for micronuclei. To describe a cytotoxic effect due to the treatment with the test substance the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and reported as the number of PCEs per 2000 erythrocytes.

The following dose levels of the test substance were investigated in male rats (6/dose):

24 h preparation interval: 500, 1000, and 2000 mg/kg b.w.

48 h preparation interval: 2000 mg/kg b.w.

The highest dose (2000 mg/kg; maximum guideline-recommended dose) was determined in a pre-experiment to be suitable, and there were no differences in toxicity between sexes. After treatment with the test substance the number of PCEs was not substantially decreased as compared to the mean value of PCEs of the vehicle control, thus indicating that bicyclopyrone did not exert any cytotoxic effects in the bone marrow.

In comparison to the corresponding vehicle controls there was no biologically relevant or statistically significant enhancement in the frequency of the detected micronuclei at any preparation interval after administration of the test substance and with any dose level used. A dose of 20 mg/kg b.w. cyclophosphamide administered orally was used as positive control, which showed a substantial increase of induced micronucleus frequency. The volume of the positive control administered was 10 mL/kg b.w.

Bicyclopyrone was negative for induction of micronuclei as determined by the micronucleus test with bone marrow cells of the rat.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirement of Test Guideline OPPTS 870.5395; OECD 474 for *in vivo* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown/beige solid
Lot/Batch number:	SEZ3AP006/Milled
Purity:	94.5%
CAS#:	352010-68-5
Stability of test compound:	Not indicated

Structure:

Control Materials:			
Negative control (if not vehicle) :	N/A	**Final Volume:** N/A	**Route:** N/A
Vehicle:	0.5% CMC	**Final Volume:** 10 mL/kg	**Route:** oral
Positive control :	Cyclophosphamide	**Final Doses:** 20 mg/kg	**Route:** oral

Test Animals:

Species	Rat
Strain	Wistar
Age/weight at acclimitisation	6-10 weeks/214.0 g (SD ± 36.4 g)
Source	Harlan Winkelmann GmbH, D-33178 Borchen
Housing	Single
Acclimatisation period	Minimum 5 days
Diet	Pelleted standard diet, ad libitum
Water	Tap water, ad libitum
Environmental conditions	Temperature: 22 ± 3 °C Humidity: 30-70% Air changes: Not specified Photoperiod: Artificial light 6.00 a.m. - 6.00 p.m.

Test compound administration:

	Dose Levels	**Final Volume**	**Route**
Preliminary:	2000 mg/kg	10mL/kg	Oral
Main Study:	2000, 1000 and 500 mg/kg	10mL/kg	Oral

Study Design and Methods:
In-life dates: Start: 19th November 2007 End: 10th December 2007

Preliminary Toxicity Assay:

A preliminary study on acute toxicity was performed in both male and female rats (two animals per sex per dose level) under identical conditions as in the main study concerning: animal strain, vehicle, route, frequency, and volume of administration.
The animals were treated orally with the test item and examined for acute toxic symptoms at intervals of around 1 h, 2-4 h, 6 h, 24 h, 30 h, and 48 h after administration of the test substance.

Micronucleus Test:

The main test was conducted using male rats only, using the following experimental design.

Experimental Design

Treatment	Dose	Number of Animals /Time of kill	
		24 hours	48 hours
CMC 0.5%	10 mL/kg	6	6
Cyclophosphamide	20 mg/kg	6	
Test substance	500 mg/kg	6	
Test substance	1000 mg/kg	6	
Test substance	2000 mg/kg	6	6

Slide Preparation: Animals were sacrificed using CO_2 followed by bleeding. The femora were removed, the epiphyses were cut off and the marrow was flushed out with foetal calf serum using a syringe. The nucleated cells were separated from the erythrocytes using the method of Romagna. Briefly, the cell suspensions were passed through a column consisting of α-Cellulose and Cellulose. The columns were then washed with Hank´s buffered saline. The cell suspension was centrifuged at 1500 rpm (390 x g) for 10 minutes and the supernatant was discarded. The pellet was resuspended in a small drop of FCS and spread on slides. The smears were air-dried and then stained with May-Grünwald /Giemsa. Cover slips were mounted with Eukitt. At least one slide was made from each bone marrow sample.

Slide Analysis: Evaluation of the slides was performed using Nikon microscopes with 100x oil immersion objectives. At least 2000 polychromatic erythrocytes (PCE) were analysed per animal for micronuclei. To describe a cytotoxic effect the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and expressed in polychromatic erythrocytes per 2000 erythrocytes. The analysis was performed with coded slides.

Five males per test group were evaluated as described. The remaining 6^{th} animal in the respective test group test group is usually evaluated in case an animal dies in its test group spontaneously.

Acceptance criteria
The study was considered valid as the following criteria are met:

- the negative controls are in the range of laboratory historical control data.
- the positive controls are in the range of laboratory historical control data.
- at least 5 animals per group could be evaluated.
- PCE to erythrocyte ratio was not less than 20 % of the negative control value.

Evaluation of results
A test substance is classified as clastogenic if it induces either a dose-related increase or a clear increase in the number of micronucleated polychromatic erythrocytes in a single dose group. Statistical methods (nonparametric Mann-Whitney test (9)) were used as an aid in

evaluating the results. However, the primary point of consideration is the biological relevance of the results.

A test substance that fails to produce a biological relevant increase in the number of micronucleated polychromatic erythrocytes is considered non-clastogenic in this system.

RESULTS AND DISCUSSION

Preliminary toxicity assay: The males treated with 2000 mg/kg b.w. exhibited minimal signs of toxicity consisting of reduced activity and ruffled fur. There were no deaths at this dose level. Females treated in the second pre-experiment exhibited clinical signs similar to those seen in males at this dose level, with no mortality.

On the basis of these data 2000 mg/kg b.w. was estimated to be suitable as the highest dose level. Since gender-specific differences in the sensitivity against the test substance were not observed, the main experiment was performed using only males.

Toxic symptoms in the main experiment: In the main experiment for the highest dose group 12 males received orally a single dose of 2000 mg/kg b.w. bicyclopyrone formulated in CMC 0.5%. The volume administered was 10 mL/kg b.w. The animals treated with 2000 mg/kg b.w. exhibited reduced activity and ruffled fur; there was no mortality at this dose level.

For the mid and low doses 6 males per group received orally a single dose of 1000 or 500 mg/kg b.w. bicyclopyrone formulated in CMC 0.5%. The volume administered was 10 mL/kg b.w. The animals treated with 1000 or 500 mg/kg b.w. expressed dose-related incidences of ruffled fur. A low incidence of reduced activity was observed in animals receiving 1000 mg/kg b.w. There were no deaths in either dose group.

The animals treated with the vehicle control (CMC 0.5%) did not express any toxic reactions.

Micronucleus test: The mean number of polychromatic erythrocytes was not decreased after treatment with the test item as compared to the mean value of PCEs of the vehicle control, indicating that bicyclopyrone did not have any cytotoxic properties in the bone marrow.

In comparison to the corresponding vehicle controls there was no biologically relevant enhancement in the frequency of the detected micronuclei at any preparation interval and dose level after administration of the test item

Summary of Micronucleus Test Results

Test group	Dose mg/kg b.w.	Sample time (h)	PCEs with micronuclei (%)	Range	PCE per 2000 erythrocytes
Vehicle	0	24	0.210	3-5	810
Test substance	500	24	0.160	2-4	985
Test substance	1000	24	0.170	2-5	982
Test substance	2000	24	0.190	1-7	1015
Positive control	20	24	2.270	24-68	916
Vehicle	0	48	0.110	1-3	961
Test substance	2000	48	0.130	0-4	1015

Table was taken from page 25 of the study report

INVESTIGATOR'S CONCLUSIONS

In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the micronucleus test with bone marrow cells of the rat. Therefore, bicyclopyrone is considered to be non-clastogenic in this micronucleus assay.

REVIEWER COMMENTS

In a Micronucleus Assay in Bone Marrow Cells of the Rat (MRID #47841984), the potential of bicyclopyrone (NOA449280) to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the rat was investigated. The test substance was formulated in 0.5%, carboxymethyl cellulose (CMC), which was also used as the vehicle control. The volume administered orally was 10 mL/kg bodyweight (b.w.). At 24 h and 48 h after a single administration of the test substance, the bone marrow cells were collected for micronuclei analysis.

In comparison to the corresponding vehicle controls there was no biologically relevant or statistically significant enhancement in the frequency of the detected micronuclei at any preparation interval after administration of the test substance and with any dose level used. A dose of 20 mg/kg b.w. cyclophosphamide administered orally was used as positive control, which showed a substantial increase of induced micronucleus frequency. The volume of the positive control administered was 10 mL/kg b.w.

Bicyclopyrone was negative for induction of micronuclei as determined by the micronucleus test with bone marrow cells of the rat.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirement of Test Guideline OPPTS 870.5395; OECD 474 for *in vivo* cytogenetic mutagenicity data. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Honavar N, 2008)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/18/15
EPA Reviewer: Greg Akerman, Ph.D. **Signature:**
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/18/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Unscheduled DNA Synthesis (UDS) with Mammalian Liver Cells *In Vivo* OECD 482 (1997): 2000/32/EEC B.39 (2000)

TEST MATERIAL (PURITY): NOA449280 (94.5% w/w)

SYNONYMS: Bicyclopyrone, SYN449280

CITATION: Clay P (2007). In Vivo Rat Liver Unscheduled DNA Synthesis Assay. Syngenta, Central Toxicology Laboratory Alderley Park, Macclesfield Cheshire, SK10 4TJ, UK. Laboratory Report No. SR1369-REG. 28 February 2007. Unpublished. (Syngenta File No. NOA449280/0039) MRID 47841983

SPONSOR: Syngenta Ltd, Alderley Park, Macclesfield, Cheshire, SK10 4TJ

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In an In Vivo Rat Unscheduled Synthesis Assay, bicyclopyrone (NOA449280) was evaluated using an autoradiographic technique, for its ability to induce unscheduled DNA synthesis (UDS) in the liver of HsdRccHan:WIST rats (3 rats/group). A single oral dose was given to groups of male rats at a dose level of 2000 mg/kg. This dose level is the limit dose level of the assay. Two sampling times, 2 hours and 16 hours post-dose were used.

The values recorded for the mean net nuclear grain counts and the percentage of cells in repair clearly show that bicyclopyrone did not induce DNA repair, as measured by UDS, at either time point investigated.

The test system positive control, N-nitrosodimethylamine (N-DMA), induced marked increases in UDS, compared to the vehicle control values, thus demonstrating the sensitivity of the test system to a known genotoxin.

Bicyclopyrone was negative for induction of DNA repair, as measured by unscheduled DNA synthesis, in the rat liver *in vivo*.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirement of Test Guideline OPPTS 870.5550; OECD 482 for a test for unscheduled DNA synthesis in mammalian cells in culture.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown/beige powder
Lot/Batch number:	SEZ3AP006
Purity:	94.5% w/w
CAS#:	352010-68-5
Stability of test compound:	Not specified

Structure:

Control Materials:

Negative control (if not vehicle) :	N/A	**Final Volume:** N/A	**Route:** N/A
Vehicle:	**1% CMC**	**Final Volume:** 20 ml/kg	**Route:** oral
Positive control :	N-nitrosodimethylamine	**Final Doses:** 10 mg/kg	**Route:** oral

Test Animals:

Species	Rats
Strain	HsdRccHan:WIST
Age/weight at dosing	6-7 weeks were used for Phase I 7-8 weeks were used for Phase II
Source	Harlan UK Ltd, Bicester, Oxon, UK
Housing	Mobile rat cage racks
Acclimatisation period	At least 5 days
Diet	Rat and Mouse No. 1 Maintenance Diet *ad libitum*
Water	Mains water *ad libitum*
Environmental conditions	Temperature: 19-25°C Humidity: 30-70% Air changes: 15/hour Photoperiod: 12 hours dark/12 hours light

Test compound administration:

	Dose Levels	**Final Volume**	**Route**
Preliminary:	2000 mg/kg	20 mL/kg	Oral
Main Study:	2000 mg/kg	20 mL/kg	Oral

Study Design and Methods:
In-life dates: Start: 16 October 2006 End: 7 December 2006

Preliminary Toxicity Assay:
Phase I involved the determination of a maximum tolerated dose (MTD), based on patterns of lethalities or severe toxicity observed over a four-day observation period following a single oral dose. A group of animals dosed at the expected MTD was also examined for micropathological changes in the liver in order to ensure that the MTD based on systemic toxicity did not induce excessive toxicity in the target organ.

UDS Assay:
Phase II, the main UDS experiments, consisted of dosing groups of rats followed by isolation and preparation of hepatocytes 2 and 16 hours post-dose. The freshly isolated hepatocytes were then cultured in the presence of tritiated thymidine and subsequently examined for UDS following autoradiography.

In Phase II, rats were weighed and given a single oral dose of CMC, N-DMA or bicyclopyrone as detailed in the following table. The bodyweights were recorded prior to dosing and are detailed below.

Group No.	Treatment	Animal Numbers	
		Experiment 1 16 hr sampling time	Experiment. 2 2 hr sampling time
		Males	Males
11	CMC	1-2	
12	NOA449280 (2000 mg/kg)	3-5	
13	N-DMA (10 mg/kg)	6-7	
14	CMC		8-9
15	NOA449280 (2000 mg/kg)		10-12
16	N-DMA (10 mg/kg)		13-14

Table was taken from page 13 of the study report

Media: Williams' E complete medium

Cell Preparation: All animals designated for perfusion were anaesthetised using halothane one at a time. Each animal was deeply anaesthetised to prevent any possible recovery. All animals treated with the test substance were examined internally for abnormalities to organs/tissues.

A 'V' shaped incision was made through both skin and muscle from the centre lower abdomen up through the rib cage. The hepatic portal vein and superior *vena cava* were cannulated with appropriate gauge catheters. The hepatocytes were isolated by a two-stage collagenase perfusion technique. A buffer solution was used to flush the liver free of blood and to remove calcium from the desmosomes. A second buffer solution to which calcium chloride and collagenase had been added was used to cause disaggregation of the liver tissue. The liver was then removed, finely chopped and filtered through a 150µm nylon bolting cloth prior to hepatocyte preparation by low speed centrifugation and resuspension in Williams' E complete medium. The viability of the hepatocytes was determined using trypan blue.

The hepatocyte suspensions were diluted with Williams' E complete medium to give a final cell count of 1.5×10^5 cells/ml and transferred onto coverslips placed etched side up in six-well plates. The cultures were placed in a humidified 37°C incubator with a 95% air: 5% CO_2 (v/v) atmosphere, for at least 90 minutes to enable cell attachment.

The culture medium was aspirated using aseptic technique and the hepatocytes washed with Williams' E incomplete medium. Williams' E incomplete medium containing ^{3}H-thymidine was added to each well and the dishes were incubated for approximately 4 hours in a 37°C incubator (humidified, 37°C, 95% air: 5% CO_2 v/v atmosphere). Cultures were then washed three times with Williams' E incomplete medium plus thymidine. This 'cold chase' procedure removed excess radiolabel from the cultures. The cultures were then incubated overnight (at least 12 hours) with the same culture medium.

The cultures were then washed once with Williams' E incomplete medium or cold chase medium prior to being fixed at least three times with freshly prepared 1:3 glacial acetic acid: absolute alcohol (v/v) followed by four washes with double deionised water. The coverslips were mounted, cell side up, on labelled microscope slides.

Preparation of Autoradiographs/Grain Development: Initially, 3 slides from each animal were coated with photographic emulsion (Ilford K2) and stored at approximately 4°C for 14 days. The spare slides were held in reserve and only processed if required.

After the exposure period, slides were developed using Kodak D19 developer and Ilford Hypam fixer. The cells were stained using Meyers Haemalum and eosin

Grain Counting: Prior to coding, the slides were examined microscopically to determine that they were of suitable quality for analysis and that they were not showing unsatisfactory levels of cytotoxicity. Slides from one vehicle control and one positive control animal were selected for UDS analysis from each experiment along with the slides from the animals treated with the test substance. The slides were coded and analysed for UDS induction using a PC based UDS data capture system.

For each cell, the number of silver grains over the nucleus [N] was determined. Then an equivalent area of cytoplasm tangential to the nucleus and with the highest apparent number of silver grains was analysed [C]. The difference between these two values [N-C] was the net nuclear grain count. Sixty cells were scored from each animal.

Data evaluation

The data were interpreted by the Study Director using concurrent and, if appropriate, historical
control data as follows:-

a) Mean net nuclear grain counts for all test substance treated animals of less than zero - negative.

b) In this laboratory no vehicle control animal has given a mean net nuclear grain count of greater than zero (Kennelly *et al*, 1995) and therefore such a value would be considered to represent a biologically significant departure from the norm. An occurrence of a mean net nuclear grain count of zero or above in a test substance treated animal is, therefore, considered to be indicative of a UDS response in that animal. Reproducibility of such a response in animals treated concurrently and in an independent experiment is necessary before concluding that the test substance is positive.

c) Where an individual animal has given rise to a mean net nuclear grain count of a least zero, but this is not fully reproducible, this may require further investigation.

d) If a test substance treated group of animals give a group mean net nuclear grain count of greater than -1, but less than 0, consideration will be given to further investigation.

RESULTS AND DISCUSSION

Phase I - Preliminary toxicity assay:

Bicyclopyrone was administered as a single oral dose to groups of male rats as detailed in the following table:

Group	Test substance	Dose (mg/kg)	Sex	Animal numbers	No. of deaths/No. dosed
1	Bicycloppyrone (NOA449280)	2000	Male	101-103	0/3

Table was taken from page 16 of the study report

Clinical Observations:

No adverse reactions to treatment were observed for animals dosed with bicyclopyrone.

Post mortem findings:
Examination of the internal organs showed no adverse findings.

No treatment related microscopic changes to the liver were observed.

Based on the findings above, the maximum tolerated dose (MTD) was considered to be in excess of 2000 mg/kg. Phase II was therefore performed using males only at a single limit dose level of 2000 mg/kg.

Phase II - UDS assay:

Clinical Observations:
No adverse reactions to treatment were observed for rats dosed with bicyclopyrone.

Post mortem findings:
Examination of the internal organs showed hardened intestines in animals treated with the test substance and positive control substance at the 16 hour sampling time.

UDS Results:

The viabilities of the hepatocyte cultures ranged from 69% to 90%.

Hepatocytes prepared from all animals were examined microscopically. No apparent signs of excessive cytotoxicity were observed on slides from animals dosed with bicyclopyrone. Slides from animals treated with bicyclopyrone were, therefore, assessed for UDS.

Bicyclopyrone caused no significant increases, compared to the vehicle control, in mean net nuclear grain count, or in percentage of cells in repair, at either time point investigated.

Hepatocytes from all bicyclopyrone treated animals had mean net nuclear grain values of less than zero. These data therefore provided no evidence for induction of UDS by bicyclopyrone.

The positive control substance, N-DMA, induced marked increases in the mean net nuclear grain counts and percentage of cells in repair.

The positive control substance, N-DMA, induced marked increases in the mean net nuclear grain counts and percentage of cells in repair.

UDS Summary data

Treatment	Dose	No. of Animals	Mean N ± SD	Mean C ± SD	Mean (N-C) ± SD	Mean % Cells in Repair
2 Hours						
1% w/v Aqueous carboxymethylcellulose (CMC)	20 mL/kg	1	4.4	7.0	-2.6	0
NOA449280	2000 mg/kg	3	4.7 ± 0.4	7.1 ± 0.3	-2.5 ± 0.2	1
N-DMA	10 mg/kg	1	15.9	5.8	10.1	75
16 Hours						
1% w/v Aqueous carboxymethylcellulose (CMC)	20 mL/kg	1	4.6	7.4	-2.8	0
NOA449280	2000 mg/kg	3	5.0 ± 0.5	8.5 ± 1.1	-3.5 ± 0.7	0
N-DMA	10 mg/kg	1	18.3	7.0	11.3	83

Data were taken from page 20 of the study report.

INVESTIGATOR'S CONCLUSIONS

Under the conditions of test, bicyclopyrone did not induce DNA repair, as measured by unscheduled DNA synthesis, in the rat liver *in vivo*.

REVIEWER'S COMMENTS

In an In Vivo Rat Unscheduled Synthesis Assay, bicyclopyrone (NOA449280) was evaluated using an autoradiographic technique, for its ability to induce unscheduled DNA synthesis (UDS) in the liver of HsdRccHan:WIST rats (3 rats/group). A single oral dose was given to groups of male rats at a dose level of 2000 mg/kg. This dose level is the limit dose level of the assay. Two sampling times, 2 hours and 16 hours post-dose were used.

The values recorded for the mean net nuclear grain counts and the percentage of cells in repair clearly show that bicyclopyrone did not induce DNA repair, as measured by UDS, at either time point investigated.

The test system positive control, N-nitrosodimethylamine (N-DMA), induced marked increases in UDS, compared to the vehicle control values, thus demonstrating the sensitivity of the test system to a known genotoxin.

Bicyclopyrone was negative for induction of DNA repair, as measured by unscheduled DNA synthesis, in the rat liver *in vivo*.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirement of Test Guideline OPPTS 870.5550; OECD 482 for a test for unscheduled DNA synthesis in mammalian cells in culture. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Clay, P 2007)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar J. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Acute Neurotoxicity Study in Rodents OECD 424 (1997): OPPTS 870.6200a (1998): 2004/73/EC B.43 (2004)

TEST MATERIAL (PURITY): NOA449280 94.5 % w/w)

SYNONYMS: NOA449280

CITATION: Beck, M., 2012. NOA449280 - An Oral (Gavage) Acute Neurotoxicity Study in Rats. WIL Research Laboratories, LLC, 1407 George Road, Ashland, OH 44805-8946, USA. Laboratory Report No. WIL-639013, issue date 23 February 2012. Unpublished. (Syngenta File No.NOA449280/11145) MRID 47842002

SPONSOR: Syngenta, Ltd. Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, United Kingdom

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In an acute neurotoxicity study in rats (MRID #47842002), bicyclopyrone (NOA449280, 94.5%) in the vehicle, 0.5% carboxymethylcellulose, was administered orally by gavage as a single dose to ten Crl:CD (SD) rats/sex/dose at dose levels of 20, 200 and 2000 mg/kg. A concurrent control group received the vehicle on a comparable regimen. Animals were approximately 6 weeks old at the initiation of dose administration. Each group consisted of 10 males and 10 females. The dose volume was 10 mL/kg for all groups.

All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily. Individual body weights were recorded weekly. Functional observational battery (FOB) and locomotor activity data were recorded for all animals prior to the initiation of dose administration, at the time of peak effect (approximately 1 hour following dose administration) on study day 0 and on study days 7 and 14. Five rats/sex/group were anesthetized on study day 15 and perfused in situ; brain weights were recorded. In addition, a neuropathological evaluation of selected tissues from the central and peripheral nervous systems was performed on 5 animals/sex in the control and 2000 mg/kg

groups. The remaining animals were euthanized on study day 15 and discarded without macroscopic examination

All animals survived to the scheduled euthanasia. There were no test substance related clinical findings during the daily observations in the test substance treated groups. Mean body weights and body weight gains were unaffected by bicyclopyrone administration. No test substance related effects were apparent in the functional observational battery evaluations.

Decreases in locomotor activity were observed for both males and females at the time of peak effect on study day 0 only, and occurred in the absence of test substance-related effects on FOB parameters and neuropathology. This transient effect on locomotor activity in the absence of any other evidence of an effect on the nervous system may be a reflection of transient systemic toxicity and is not considered adverse.

Based upon the effects in this study, the NOAEL for neurotoxicity is 2000 mg/kg. The LOAEL was not observed.

Based upon the effects in this study, the NOAEL for systemic toxicity is 2000 mg/kg. The LOAEL was not observed.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the requirement (EPA OPPTS 870.6200; OECD 424) for an acute neurotoxicity screening study in rats.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown beige powder
Lot/Batch number:	SEZ3AP0061MfLLED
Purity:	94.5 % w/w a.i.
CAS#	352010-68-5
Stability of test compound:	End March 20122 (stored at room temperature, away from direct sunlight)

Structure:

Vehicle and/or positive control: 0.5% w/v aqueous carboxymethylcellulose (CMC)

Test Animals:

Species	Rat
Strain	Crl:CD(SD) rats
Age/weight at dosing	Approximately 6 weeks / males 182-233g, females 141-190g.
Source	Charles River Laboratories, Inc., Raleigh, NC
Housing	Individually in suspended stainless steel, wire-mesh cages
Acclimatisation period	Approximately 14 days
Diet	PMI LLC Certified Rodent LabDiet 5002 (meal) ad libitum
Water	Reverse osmosis treated municipal water ad libitum
Environmental conditions	Temperature: 21.5-22. Humidity: 37.0-48.5% Air changes: A minimum of 10 changes/hour Photoperiod: 12 hours light / 12 hours dark

Study Design and Methods:

In-life dates: Start: 25 March 2008 End: 03 June 2008

Dose Selection rationale: The selected doses were based upon the results from an acute range-finding study (MRID #47842151, see appendix).

Animal assignment and treatment: The study design is depicted in table 1. Animals were randomly assigned to the four test groups using computer-generated random algorithm. Each group (Groups 1-4) consisted of 10 males and 10 females. These animals were then randomized into 4 study replicates to allow for the reasonable conduct of the functional observational battery and locomotor activity assessments.

The rats were dosed once on day 1 by oral gavage at a dose volume of 10 mL/kg according to their individual bodyweights at the time of dosing. Control animals received the vehicle (0.5% w/v aqueous carboxymethylcellulose (CMC)), only. Dose levels were selected based on the results of a preliminary acute neurotoxicity study of bicyclopyrone in rats. In that study, there were no remarkable clinical findings noted during the clinical examinations and there were no test substance-related effects on body weights or body weight changes. Therefore, dose levels of 20, 200 and 2000 mg/kg were selected for the current study.

At the end of the scheduled period, 5 rats/sex/group were perfused *in situ*. Selected nervous system tissues were removed, and preserved in an appropriate fixative. From the five animals/sex/group killed by perfusion fixation, the brain was removed and the weight recorded. The remaining animals were killed and discarded. Submitted tissues from top dose and control animals were examined by light microscopy.

Table 1: Study design

Experimental Parameter	Dose Group (mg/kg)			
	0 (control)	20	200	2000
Total number of animals/group	10/sex	10/sex	10/sex	10/sex
Behavioural testing (FOB, Motor Activity)	10/sex	10/sex	10/sex	10/sex
Neuropathology	5/sex	5/sex	5/sex	5/sex

Table was taken from page 20 of the study report

Test substance preparation and analysis: The appropriate amount of the test substance for each formulation was weighed into a calibrated glass container. Approximately 70% of the vehicle (0.5% (w/v) CMC) was added to each container and the formulations were mixed using a magnetic stirrer until the test substance was wetted. Vehicle was then added to each container to bring the formulations to the calibration mark. The formulations were homogenized for approximately 5 minutes and then stirred with a magnetic stirrer until a uniform mixture was obtained

The test substance formulations were prepared once as single formulations for each dose level, divided into aliquots for daily dispensation and stored refrigerated. The test substance formulations were stirred continuously throughout the preparation, sampling and dose administration procedures.

Results

Stability: The test substance, at a concentration range of 1 to 200 mg/mL, was determined to be stable for up to 5 days under refrigerated conditions (WIL-639012); therefore, stability was not assessed as a part of this study.

Homogeneity analysis: Duplicate samples from the top, middle and bottom strata of the formulations prepared at target concentrations of 2, 20, and 200 mg NOA449280/mL were analysed to assess test substance homogeneity. The results of the homogeneity analyses are presented in Table AC1, with the overall statistics summarized in the following table. The analyzed formulations met the WIL standard operating procedure requirements for test substance homogeneity, i.e., the RSD for the mean concentration was 10% or less at a concentration within the acceptable limits (85% to 115% of the target concentration).

Concentration analysis: The analyzed formulations met the WIL Research SOP requirements for homogeneity, i.e., the RSD for the mean concentration was ≤10% at a concentration within the acceptable limits (85% to 115% of the target concentration), and for concentration acceptability in suspension formulations, i.e., the mean concentration was 85% to 115% of the target concentration.

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable, provided that the cited stability study did indicate that the test compound was stable under conditions of the study.

Mortality and clinical observations: Observations for viability / mortality were recorded twice daily. All animals were observed for clinical signs once during acclimatisation, at approximately 1 hour after dose administration on day 1 and once daily thereafter. In addition, detailed clinical observations were made. The animals were observed in their home cages, outside their home cages in a standard arena and in the hand. These observations were performed in random sequence once before commencement of administration, at approximately 5 hours after dosing on day 0, and on days 7 and 14.

Bodyweight: Body weights were recorded once during the acclimatisation period and on days 0, 7 and 14.

Food consumption: Food consumption was not recorded.

Cholinesterase determination: Cholinesterase activity was not determined in this study.

Neurobehavioural Assessment:

Functional observational battery (FOB): Functional observational battery (FOB) findings were recorded for all animals 6 days prior to the initiation of dose administration, at the time of peak effect (1 hour post dosing) on study day 0 and on study days 7 and 14. Evaluations included a quantitative assessment of landing foot splay, sensory perception and muscle weakness. For the conduct of the FOB, animals were randomised and the cage labels covered with the corresponding FOB number in order to make experimenters unaware of the animal's treatment group.

Animals were observed in their home cage, during handling and in an open field. Observations were conducted over the functional domains of CNS activity, CNS excitation and sensorimotor, autonomic and physiological functions. Functional examinations included tests for: Sensorimotor Functions (approach, touch, vision, audition, pain, vestibular); Autonomic Functions (pupillary reflex, body temperature) and Sensorimotor Coordination (grip strength, fore- and hind-paws, and landing foot splay).

Locomotor activity: Locomotor activity was assessed for all animals 6 days prior to the initiation of dose administration, at the time of peak effect (1 hour post-dosing) on study day 0 and on study days 7 and 14. Locomotor activity, recorded after the completion of the FOB, was measured automatically using the Kinder Scientific Motor Monitor System (Kinder Scientific, LLC, Poway, CA). This personal computer-controlled system utilized a series of infrared photobeams surrounding a clear plastic, rectangular cage to quantify the motor activity of each animal. Four-sided black plastic enclosures were used to surround the clear plastic boxes and decrease the potential for distraction from extraneous environmental stimuli or activity by technicians or adjacent animals. The black enclosures rested on top of the photobeam frame and did not interfere with the path of the beams. The locomotor activity assessment was performed in a sound-attenuated room equipped with a white-noise generator set to operate at 70 ± 10 dB. The testing of treatment groups was conducted according to replicate sequence. Each animal was tested separately. Data were collected in 5-minute epochs, and the test session duration was 60 minutes. These data were compiled as six 10-minute subsessions for tabulation. Data for ambulatory and total motor activity were tabulated. Total motor activity was defined as a combination of fine motor skills (i.e., grooming, interruption of 1 photobeam) and ambulatory motor activity (interruption of 2 or more consecutive photobeams).

Termination and pathology: On study day 15, 5 rats/sex/group were anesthetized by an intraperitoneal injection of sodium pentobarbital and then perfused in situ with a buffered 4.0% paraformaldehyde/ 1.4% glutaraldehyde solution. The central and peripheral nervous system tissues were dissected and preserved. The fixed brain weight (including olfactory bulbs) was recorded. Any observable gross changes, abnormal coloration or lesions of the brain and spinal cord were recorded. The remaining rats were euthanized by carbon dioxide inhalation and discarded without macroscopic examination. The following nerve tissues were prepared for a microscopic neuropathologic examination from all selected animals per sex in the control and 2000 mg/kg groups:

Brain - olfactory bulbs, cerebral cortex (2 levels), hippocampus/dentate gyrus, basal ganglia, thalamus, hypothalamus, midbrain (tectum, tegmentum and cerebral peduncles), cerebellum, pons and medulla oblongata

Spinal cord - at cervical swellings C3-C7 and at lumbar swellings T13-L4

Trigeminal ganglia/nerves

Lumbar dorsal root ganglia at T13-L4

Lumbar dorsal root fibers at T13-L4

Lumbar ventral root fibers at T13-L4

Cervical dorsal root ganglia at C3-C7

Cervical dorsal root fibers at C3-C7

Cervical ventral root fibers at C3-C7

Sciatic nerves (mid-thigh region)

Sciatic nerves (at sciatic notch)

Sural nerves

Tibial nerves

Peroneal nerves

Eyes with Optic nerves

Skeletal muscle (gastrocnemius)

All submitted tissues from control and high dose group animals were processed unless indicated for storage. The central nervous system tissues listed above were prepared for the qualitative histopathological examination by embedding in paraffin, sectioning and staining with hematoxylin and eosin. The peripheral nervous tissues listed in this section were prepared for examination by embedding in plastic, sectioning and staining using methodology deemed appropriate by the pathologist.

All submitted tissues from top dose and control animals, except those indicated for storage, were examined by light microscopy.

Statistics: Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test substance-treated group to the control group by sex. Body weights, body weight changes, continuous functional observational battery data, locomotor activity data, absolute brain weights and terminal body weights were analysed by a parametric one-way analysis of variance (ANOVA) (Snedecor and Cochran, 1980) and Dunnett's test (Dunnett, 1964). In addition, brain weights were analysed by analysis of covariance on final body weight. FOB parameters that yielded scalar or descriptive data, necropsy findings and non-graded histopathologic findings were analysed using Fisher’s Exact Test (Steel and Torrie, 1980). Graded histopathologic findings with multiple severities (i.e., those findings with more than 2 distinct severities including none/not remarkable) were compared to the control groups using the Mann-Whitney U-test (Kruskal and Wallis, 1952). Analyses of absolute brain weight, terminal body weight and graded histopathological findings were conducted by BioSTAT Consultants, Inc., Portage, MI. No statistical analysis was performed for organ to body weight ratios as the analysis of covariance provides a better method of allowing for differences in final body weight (Shirley, 1977).

RESULTS AND DISCUSSION

Clinical signs and mortality: There were no deaths and no treatment-related clinical observations.

Body weight and body weight gain: Body weights and body weight gains were unaffected by test substance administration. Absolute body weights for both sexes are presented in table 2.

Table 2: Mean intergroup comparison of absolute bodyweights (g) by week

	Dietary Concentration of bicyclopyrone (mg/kg)							
	Males				Females			
Week*	**0**	**20**	**200**	**2000**	**0**	**20**	**200**	**2000**
Day -6	142 ± 2.6	140 ± 5.7	138 ± 6.5	135 ± 4.2	130 ± 4.2	130 ± 4.2	131 ± 3.5	125 ± 7.5
Day 0	209 ± 9.1	209 ± 11.3	209 ± 12.6	210 ± 17.7	163 ± 7.6	168 ± 10.3	165 ± 9.8	164 ± 11.3
Day 7	264 ± 8.2	264 ± 14.3	259 ± 18.7	263 ± 21.6	185 ± 13.4	189 ± 15.7	183 ± 12.8	184 ± 14.0
Day 14	309 ± 12.6	312 ± 17.3	309 ± 25.8	313 ± 27.2	206 ± 13.1	210 ± 14.3	202 ± 13.1	208 ± 18.1

Data were taken from pages 37-38 of the study report

Neurobehavioural Assessment:

Functional observational battery (FOB): There were no test substance-related effects on FOB (functional observational battery) evaluations performed at the time of peak effect (approximately 1 hour post-dosing) on study day 0 or on study days 7 and 14.

Motor activity: The only test substance-related finding in this study was a decrease in locomotor activity at 2000 mg/kg on study day 0. When evaluated at the time of peak effect (approximately 1 hour following dose administration), locomotor activity in the 2000 mg/kg group males was 35.8% (total) and 50.0% (ambulatory) lower than that of the control group for the overall 60-minute test session, primarily as a result of significantly decreased activity during the first 20 minutes of the test session. The 2000 mg/kg group females also had decreased ambulatory activity during the first 10 minutes of the test session on study day 0; however, the reduction (21.8%), while significant, was not sufficient to affect the overall test session. Mean total counts were unaffected for females at this dose level. These changes in locomotor activity occurred in the absence of test substance-related effects on FOB parameters at the time of peak effect on study day 0 and did not persist to the locomotor activity evaluations on study days 7 and 14. Furthermore, there were no neuropathologic changes that were attributed to the test substance. Motor activity data are presented in table 3.

Table 3: Selected Motor activity findings

	Dose of bicyclopyrone (mg/kg)							
	Males				Females			
	0	**20**	**200**	**2000**	**0**	**20**	**200**	**2000**

0-60 minutes (mean)	2638 ± 1107	1787*± 492.4	2237 ± 585.6	1694* ± 653.1	2596 ± 771.3	2190 ± 694.2	2379 ± 912.0	2199 ± 580.2
% difference		-32.3	-15.2	-35.8		-15.6	-8.4	-15.3
Ambulatory	582± 268.7	334**± 103.5	457 ± 163.0	291** ± 126.9	653 ± 315.8	571± 276.6	589 ± 243.0	484 ± 233.9
% difference	-	-42.6	-21.5	-50.0	-	-12.6	-9.8	-25.9

Data taken from pages 126 and 144
*statistically different from control at 0.05 (Dunnett's test)
**statistically different from control at 0.01 (Dunnett's test)

Sacrifice and pathology:

Gross pathology: There were no gross lesions recorded at necropsy.

Brain weight: There were no treatment-related effects on brain weight.

Neuropathology: In the control and high-dose groups, axonal degeneration was noted in spinal root fibres and peripheral nerves but was of minimal severity in all instances and was considered spontaneous. Spontaneous axonal degeneration has been reported as the most frequent lesion in the central and peripheral nervous systems of both control and treated rats (Eisenbrandt et al., 1990).

INVESTIGATOR'S CONCLUSIONS: Based on a transient decrease in locomotor activity in males and females at 2000 mg/kg, the no-observed-effect level (NOEL) for a single oral dose of bicyclopyrone in rats is concluded to be 200 mg/kg.

REVIEWER COMMENTS:

Based upon the effects in this study, the NOAEL for neurotoxicity is 2000 mg/kg. The LOAEL was not observed.

Based upon the effects in this study, the NOAEL for systemic toxicity is 2000 mg/kg. The LOAEL was not observed.

PMRA (Canada) believes that the decreased locomotor activities at the high dose are adverse and that the LOAEL is 2000 mg/kg. EPA and APVMA/OCS (Australia) don't consider this effect adverse.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the requirement (EPA OPPTS 870.6200a; OECD 424) for an acute neurotoxicity screening study in rats. PMRA disagrees with EPA and APVMA/OCS (Australia) regarding the scientific conclusion of this study but agrees regarding its regulatory decision and classification.

(Beck M., 2012)

Appendix

STUDY TYPE: Preliminary Acute Neurotoxicity – oral gavage - rats (No applicable test guidelines)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one.

CITATION: Beck M, 2009. A preliminary acute neurotoxicity study of bicyclopyrone technical in rats. WIL Research Laboratories, Ashland, USA. Laboratory Report No. WIL-639012, 19 August 2009. Unpublished. (Syngenta File No. NOA449280/11211) MRID 47842151

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY UK.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

EXECUTIVE SUMMARY

A preliminary range-finding acute neurotoxicity study (MRID #47842151) bicyclopyrone (NOA449280) was conducted to select dose levels for use in a definitive acute neurotoxicity study and to determine the appropriate time following dose administration at which neurobehavioral endpoints should be assessed. Groups of three male and three female, approximately 6 week old, Crl:CD(SD) rats were administered single oral gavage doses of 0 (control) or 2000 mg bicyclopyrone mg/kg bw in 0.5% w/v aqueous carboxymethylcellulose (CMC).

All animals were observed twice daily for mortality and moribundity. Clinical observations and body weights were recorded at appropriate intervals. Modified functional observational battery (FOB) data were recorded for all animals at approximately 1, 2, 3, 4, 5, 6, 7 and 8 hours following dose administration on study day 0 and once daily on study days 1-7. On study day 8, all animals were killed by carbon dioxide inhalation and discarded without macroscopic examination.

All animals survived to the scheduled euthanasia. No remarkable clinical findings were noted during the clinical examinations. There were no test substance-related effects on body weights or body weight changes. Modified functional observational battery parameters evaluated at 1, 2, 3, 4, 5, 6, 7 and 8 hours following dose administration on study day 0 and daily on study days 1-7 were unaffected by dose administration.

Based upon the effects in this study, the NOAEL for neurotoxicity is 2000 mg/kg. The LOAEL was not observed.

Based upon the effects in this study, the NOAEL for systemic toxicity is 2000 mg/kg. The LOAEL was not observed.

Based on the absence of test substance-related effects on survival, clinical condition, body weight or modified functional observational battery parameters at 2000 mg/kg bw, dose levels of 20, 200 and 2000 mg/kg bw were selected for a definitive acute neurotoxicity study of bicyclopyrone technical administered orally by gavage to Crl:CD (SD) rats. The high dose was selected as it represents the limit dose as defined by U.S. EPA OPPTS Guideline 870.6200. Based on the lack of findings noted at any time point following dose administration, 1 hour post-dose was selected as the time of peak effect for study day 0 FOB and locomotor activity evaluations.

This study is classified as totally reliable (**acceptable/non-guideline**) and satisfies the requirement (EPA OPPTS 870.6200; OECD 424) for an acute neurotoxicity screening range-finding study in rats.

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anw Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) Date: 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) Date: 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Neurotoxicity Study in Rodents OECD 424 (1997): OPPTS 870.6200b (1998): 2004/73/EC B.43 (2004)

TEST MATERIAL (PURITY): NOA449280 94.5 % w/w)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Beck, M., 2012. NOA449280- 90-Day Dietary Neurotoxicity Study in Rats. WIL Research Laboratories, LLC, 1407 George Road, Ashland, OH 44805-8946, USA. Laboratory Report No. WIL-639017, issue date 29 February 2012. Unpublished. (Syngenta File No.NOA449280/11146) MRID 47842004

SPONSOR: Syngenta, Ltd. Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, United Kingdom

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a subchronic dietary neurotoxicity study (MRID #47842004), groups of 12 Crl:CD (SD) rats/sex were fed bicyclopyrone (NOA449280, 94.5%) admixed to their diet for 13 weeks at concentrations of 0, 50, 500 and 5000 ppm (0, 4/4, 35/42, and 336/415 mg/kg/day [M/F]). General cage side observations were made for all animals prior to study start and daily throughout the study. Detailed clinical observations comprising open field evaluation of clinical signs were performed in a randomised order once prior to initiation of treatment and once weekly thereafter. Functional observation batteries (FOBs) including quantitative assessments of landing foot splay, sensory perception and muscle weakness were performed for all animals in a randomised order once prior to initiation of treatment, and in weeks 2, 4, 8 and 13. Locomotor activities were assessed after each FOB evaluation. Food consumption values were recorded weekly throughout the study. Body weights were recorded once prior to initiation of treatment on day 1 and weekly thereafter. Ophthalmic examinations were performed for all animals prior to initiation of treatment and during week 11. During study week 13, 5 rats/sex/group were anesthetized and perfused in-situ; brain weights were recorded and neuropathological evaluation of selected tissues from the central and peripheral nervous

systems was performed on rats in the control and 5000 ppm groups. The remaining animals were euthanized by carbon dioxide inhalation during study week 13 and discarded without macroscopic examination.

At all doses, there were statistically significant decreases in the absolute brain weights in males (↓8-11%), which were within the historical control range and were not considered adverse.

Effects that were considered treatment-related and adverse are as follows:

At 50 ppm bicyclopyrone, there was a treatment-related unilateral or bilateral keratitis observed for males (17%).

At 500 ppm bicyclopyone, there was a treatment-related unilateral or bilateral keratitis observed for males (25%).

At 5000 ppm bicyclopyrone, there was a treatment-related unilateral or bilateral keratitis observed for males (25%) and one female (8%). Reduced absolute body weights were observed for both sexes (↓11-12% for males and ↓11-14% for females).

Based upon the effects of this study, the systemic LOAEL is 50 ppm (4 mg/kg/day) based upon an increased incidence of unilateral keratitis in the eyes of males. The systemic NOAEL was not observed.

Neurotoxicity NOAEL = 5000 ppm (336/415 mg/kg/day [M/F]). Neurotoxicity LOAEL was not observed.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirements (OPPTS 870.6200; OECD 424) for a subchronic neurotoxicity screening study in rats.

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, Flagging and Quality Assurance statements were provided.

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Brown beige powder
Lot/Batch number:	SEZ3AP006/MILLED
Purity:	94.5 % w/w a.i.
CAS#	352010-68-5
Stability of test compound:	End March 2012 (stored at room temperature, away from direct sunlight)

Structure:

Vehicle and/or positive control: LLC Certified Rodent LabDiet 5002

Test Animals:

Species	Rat
Strain	Crl:CD(SD)
Age/weight at dosing	Six weeks old at start of treatment. 170-225 g (males) / 124-180 g (females)
Source	Charles River Laboratories, Inc., Raleigh, NC
Housing	Individually in suspended stainless steel, wire-mesh cages
Acclimatisation period	Approximately 6 days
Diet	Certified Rodent LabDiet® 5002, ad libitum
Water	Reverse osmosis; ad libitum.
Environmental conditions	Temperature: 19.1-23.0 °C Humidity: 33.6-52.5 % Air changes: A minimum of 10 changes/hour Photoperiod: 12 hours light / 12 hours dark

Study Design and Methods:

In-life dates: Start: 20 January 2009 End: 22 April 2009

Dose Selection rationale: The selected doses were based upon the results from an acute range-finding study (MRID #47842140, see appendix).

Animal assignment and treatment: Animals were randomly assigned to the four test groups using computer-generated random algorithm. Each group (Groups 1-4) consisted of 12 males and 12 females. The rats were fed bicyclopyrone admixed to their diet for 13 weeks at concentrations of 0, 50, 500 and 5000 ppm. Control animals received basal diet only.

At the end of the scheduled period, 5 rats/sex/group were perfused *in situ*. Selected nervous system tissues were removed, and preserved in an appropriate fixative. From the five animals/sex/group killed by perfusion fixation, the brain was removed and the weight recorded. The remaining animals were killed and discarded. Submitted tissues from top dose and control animals were examined by light microscopy.

Table 1: Study design

Group Number	Treatment	Dose Concentration (ppm)	Number of Males	Number of Females
1	Basal diet	0	12	12
2	NOA449280	50	12	12
3	NOA449280	500	12	12
4	NOA449280	5000	12	12

Table was taken from page 21 of the study report

Test substance preparation and analysis: The diets were prepared as test substance/diet mixtures using an appropriate blender. All test diets were prepared approximately every 2 weeks. All diets were based on PMI Nutrition International, LLC Certified Rodent LabDiet® 5002. Following preparation, the test diets were placed in appropriate storage bags and stored at room temperature until use.

Stability analysis: Stability of the dietary preparations at concentrations ranging from 25 ppm to 5000 ppm was established for up to 15 days of room temperature and frozen storage (WIL-639016). Therefore, stability assessments were not conducted as part of this study.

Homogeneity analysis: Duplicate samples from the top, middle and bottom strata of the formulations prepared at nominal concentrations of 50, 500 and 5000 ppm bicyclopyrone were analyzed to assess test substance homogeneity. Duplicate samples from the middle stratum of the basal diet were also collected and analyzed. The analyzed formulations met the WIL standard operating procedure requirements for test substance homogeneity, i.e., the RSD for the mean concentration was 10% or less at a concentration within the acceptable limits (85% to 115% of the target concentration).

Concentration analysis: Formulations used for dose administration were analyzed to assess test substance concentration acceptability. The analyzed formulations used for test substance administration met the WIL Research SOP requirement for concentration acceptability for diet admix formulations, i.e., the analysed concentration was 85% to 115% of the target concentration.

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable, provided that the cited stability study did indicate that the test compound was stable under conditions of the study.

Mortality and clinical observations: All animals were observed twice daily, once in the morning and once in the afternoon, for mortality and moribundity. Clinical observations were recorded weekly, beginning approximately 1 week prior to test diet administration and continuing until the scheduled necropsy.

Bodyweight: Individual body weights were recorded weekly, beginning approximately 1 week prior to test diet administration.

Food consumption: Individual food consumption was recorded weekly, beginning approximately 1 week prior to test diet administration.

Ophthalmoscopy: Ocular examinations were conducted on all animals prior to the initiation of test diet administration (study week -2) and near the end of the treatment period (study week 11). All ocular examinations were conducted using an indirect ophthalmoscope and slit lamp biomicroscope (or other suitable equivalent equipment) preceded by pupillary dilation with an appropriate mydriatic agent.

Cholinesterase determination: Cholinesterase activity was not determined in this study.

Neurobehavioural Assessment:

Functional observational battery (FOB): Functional observational battery (FOB) findings were recorded for all animals during pretest (study week -1) and during study weeks 1, 3, 7, and 12. Evaluations included quantitative assessments of landing foot splay, sensory perception and muscle weakness. For the conduct of the FOB, animals were randomised and the cage labels covered with the corresponding FOB number in order to make experimenters unaware of the animal's treatment group.

Animals were observed in their home cage, during handling and in an open field. Observations were conducted over the functional domains of CNS activity, CNS excitation and sensorimotor, autonomic and physiological functions. Functional examinations included tests for sensorimotor functions (approach, touch, vision, audition, pain, vestibular), autonomic functions (pupillary reflex, body temperature) and sensorimotor coordination (grip strength (fore- and hind-limb) and landing foot splay).

Locomotor activity: Locomotor activity was assessed for all animals during pretest (study week -1) and during study weeks 1, 3, 7, and 12. Locomotor activity, recorded after the completion of the FOB, was measured automatically using the Kinder Scientific Motor Monitor System (Kinder Scientific, LLC, Poway, CA). This personal computer controlled system utilized a series of infrared photobeams surrounding a clear plastic, rectangular cage to quantify the motor activity of each animal. Four-sided black plastic enclosures were used to surround the transparent plastic boxes and decrease the potential for distraction from extraneous environmental stimuli or activity by technicians or adjacent animals. The black enclosures rested on top of the photobeam frame and did not interfere with the path of the beams. The locomotor activity assessment was performed in a sound attenuated room equipped with a white noise generator set to operate at 70 ± 10 dB. The testing of treatment groups was conducted according to replicate sequence. Each animal was tested separately. Data were collected in 5 minute epochs, and the test session duration was 60 minutes. These data were compiled as six 10-minute subintervals for tabulation.

Data for ambulatory and total motor activity were tabulated. Total motor activity was defined as a combination of fine motor skills (i.e., grooming, interruption of 1 photobeam) and ambulatory motor activity (interruption of 2 or more consecutive photobeams).

Termination and pathology: At study termination (study week 13), 5 randomly selected rats/sex/group were anesthetized by an intraperitoneal injection of sodium pentobarbital and then perfused in situ with a buffered 4.0% paraformaldehyde/1.4% glutaraldehyde solution. The central and peripheral nervous system tissues were dissected and preserved. Fixed brain weight (including olfactory bulbs) was recorded. Any observable gross changes, abnormal coloration, or lesions of the brain and spinal cord were recorded. The remaining rats were euthanized by carbon dioxide inhalation and discarded without macroscopic examination. The following nerve tissues were prepared for a microscopic neuropathologic examination from the 5 rats/sex selected for neuropathology in the control and 5000 ppm groups:

Brain - olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, midbrain, cerebellum, pons, and medulla oblongata

Spinal cord - at cervical swellings C3-C7 and at lumbar swellings T13-L4

Trigeminal ganglia/nerves

Lumbar dorsal root ganglia at T13-L4

Lumbar dorsal root fibers at T13-L4

Lumbar ventral root fibers at T13-L4

Cervical dorsal root ganglia at C3-C7

Cervical dorsal root fibers at C3-C7

Cervical ventral root fibers at C3-C7

Sciatic nerves (mid-thigh region)

Sciatic nerves (at sciatic notch)

Sural nerves

Tibial nerves

Peroneal nerves

Eyes with Optic nerves

Skeletal muscle (gastrocnemius)

All submitted tissues from control and high dose group animals were processed unless indicated for storage. The central nervous system tissues listed above were prepared for the qualitative histopathological examination by embedding in paraffin, sectioning, and staining with hematoxylin and eosin. The peripheral nervous tissues listed in this section were prepared for examination by embedding in plastic, sectioning, and staining using methodology deemed appropriate by the pathologist.

All submitted tissues from top dose and control animals, except those indicated for storage, were examined by light microscopy.

Statistics: All statistical tests were performed using appropriate computing devices or programs. Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test substance-exposed group to the control group by sex.

Body weight, body weight change, cumulative body weight change, food consumption, pretest and weekly continuous functional observational battery (FOB) data, locomotor activity data, and absolute brain weights were subjected to a parametric one-way analysis of variance (ANOVA). Brain weights were also analysed by analysis of covariance on final body weight. FOB parameters that yielded scalar or descriptive data, and non-graded histopathologic findings were analysed using Fisher's Exact Test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Clinical signs and mortality: One female in the 5000 ppm group was euthanized in extremis on study day 42. Clinical findings for this female were decreased defecation, misaligned upper incisors and red material around the eyes on the day of euthanasia. This female lost 74 g of body weight during study days 35-42 and had slightly decreased food consumption during this period. The clinical findings were consistent with a mechanical injury, and no relationship to the test substance was apparent. All other animals survived to the scheduled euthanasia, with no treatment related clinical observations seen.

Body weight and body weight gain: Absolute body weight and body weight gain data are presented in tables 2 and 3. Mean absolute body weights for the 5000 ppm group males and females were statistically significantly lower when compared with the control group throughout the study (↓11-12% for males and ↓11-14% for females). Mean body weight gains for the 5000 ppm group males and females were statistically significantly lower when compared with the control group during study week 0-1. Mean overall (weeks 1-13) decreases in body weight gains were 19% (males) and 26% (females) lower when compared with the control group throughout the study.

Statistically significant changes in mean body weight gain and cumulative body weight gain noted for the 50 and 500 ppm females were considered sporadic and were not considered test substance-related.

Table 2: Mean intergroup comparison of absolute bodyweights (g) by week

	Dietary Concentration of bicyclopyrone (ppm)							
	Males				Females			
Week*	**0**	**50**	**500**	**5000**	**0**	**50**	**500**	**5000**
Pre-Test (Week 0)	199 ± 11.2	199 ± 11.8	198 ± 14.3	201 ± 11.2	151 ± 8.8	153 ± 14.3	158 ± 10.1	151 ± 12.6
Week 1	260 ± 15.4	257 ± 13.4	256 ± 20.2	246 ± 16.4	174 ± 12.5	170 ± 13.9	178 ± 13.4	165 ± 16.7
Week 2	310 ± 22.8	304 ± 17.8	300 ± 26.0	288 ± 24.3	198 ± 14.3	192 ± 16.6	201 ± 16.3	186 ± 18.4
Week 3	354 ± 30.9	348 ± 23.3	342 ± 27.6	327 ± 34.2	214 ± 14.8	207 ± 18.0	215 ± 19.3	197 ± 20.4
Week 6	445 ± 47.3	431 ± 41.6	425 ± 35.7	406± 47.3	256 ± 20.9	244 ± 19.9	254 ± 25.0	224** ± 22.0 (↓13%)
Week 7	471 ± 50.2	452 ± 45.7	448 ± 36.5	425 ± 52.8	267 ± 21.9	252 ± 19.4	265 ± 26.0	231** ± 20.1 (↓14%)
Week 9	513 ± 54.0	493 ± 52.3	487 ± 40.8	458* ± 54.0 (↓11%)	277 ± 20.5	265 ± 19.7	281 ± 33.1	246** ± 20.1 (↓11%)
Week 13	565 ± 62.9	546 ± 59.6	531 ± 48.5	497* ± 60.5 (↓12%)	304 ± 34.7	284 ± 21.7	301 ± 33.4	262** ± 24.3 (↓14%)

Data were taken from pages 43-50 of the study report

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Table 3: Intergroup comparison of bodyweight gain (g)

Study Days	Dose level of bicyclopyrone (ppm)							
	Males				Females			
	0 (control)	**50**	**500**	**5000**	**0 (control)**	**50**	**500**	**5000**
0-7	61 ± 6.0	58 ± 5.5	58± 8.2	45** ± 9.7 (↓26%)	23 ± 7.4	16* ± 5.6	21± 5.6	14** ± 7.5 (↓39%)
0-21	155 ± 25.1	149 ± 19.5	143 ± 17.8	126* ± 31.2 (↓19%)	63± 10.8	53 ± 10.5	57 ± 12.0	46** ± 12.4 (↓27%)
0-42	246 ± 41.1	232 ± 39.0	227 ± 26.9	205*± 45.2 (↓17%)	105 ± 15.6	91 ± 10.6	97 ± 10.9	73** ± 21.1 (↓30%)

0-70	332 ± 54.1	312 ± 55.0	307 ± 32.9	275*± 55.1 (↓17%)	137 ± 19.9	120 ± 14.1	130 ± 23.7	101*± 15.8 (↓26%)
0-91	366 ± 57.3	347 ± 58.0	333 ± 41.4	296* ± 57.5 (↓19%)	153 ± 30.3	131 ± 16.5	143 ± 28.7	113**± 19.8 (↓26%)

Data were taken from pages 57-62 of the study report
*statistically significantly different from control at 0.05 (Dunnett's test)
**statistically significantly different from control at 0.01 (Dunnett's test)

Food consumption and test substance intake: Mean food consumption for the 5000 ppm group males was slightly lower (24 g/animal/day) (statistically significant) when compared with the control group (27 g/animal/day) during study week 0-1 and generally continued to be slightly lower (rarely statistically significant) than the control group throughout the remainder of the exposure period. See table 4.

The mean calculated test substance consumption was 4, 35 and 336 mg/kg/day (males) and 4, 42 and 415 mg/kg/day (females) for the 50, 500 and 5000 ppm groups respectively.

Table 4: Intergroup comparison of food consumption (g/rat/day); selected time points

Study Days	Dose level of bicyclopyrone (ppm)							
	Males				Females			
	0 (control)	50	500	5000	0 (control)	50	500	5000
0-7	27 ± 1.9	26 ± 1.5	26 ± 2.0	24** ± 2.1 (↓11%)	19 ± 1.7	17 ± 0.8	20 ± 3.3	17± 2.1
14-21	29 ± 3.0	30 ± 2.7	30 ± 2.4	27 ± 3.0	20 ± 2.0	19 ± 1.8	21 ± 2.3	20 ± 3.0
28-35	29 ± 2.8	28 ± 3.0	28 ± 5.7	27 ± 2.7	20 ± 2.0	20 ± 1.2	21 ± 2.0	19 ± 1.6
56-63	29 ± 2.7	29 ± 2.7	28 ± 1.9	26* ± 3.1 (↓10%)	19 ± 1.2	19 ± 0.5	21 ± 2.9	18 ± 1.7
84-91	28 ± 2.0	28 ± 2.6	27 ± 2.5	25* ± 4.3 (↓11%)	19 ± 2.3	19 ± 2.5	21 ± 3.4	18 ± 1.6

Data were taken from pages 63 to 74 of the study report
*statistically significantly different from control at 0.05 (Dunnett's test)
**statistically significantly different from control at 0.01 (Dunnett's test)

Ophthalmic Examination: Treatment-related unilateral or bilateral keratitis was observed for the 50, 500, and 5000 ppm group males (17-25%) and a single unilateral occurrence was observed for a 5000 ppm group female. Data are presented in table 5.

Table 5: Intergroup comparison of selected ophthalmic findings at week 11

Finding	Dose level of bicyclopyrone (ppm)							
	Males				Females			
	0 (control)	50	500	5000	0 (control)	50	500	5000
Keratitis, unilateral	0	2 (17%)	3 (25%)	2 (17%)	0	0	0	1 (8%)
Keratitis, bilateral	0	1 (8%)	0	0	0	0	0	0

Data were taken from pages 1176-1184 of the study report

Neurobehavioural Assessment:

Functional observational battery (FOB): There were no test substance-related effects on FOB (functional observational battery) evaluations performed at the time of peak effect (approximately 1 hour post-dosing) during study weeks 1, 3, 7 and 12.

Motor activity: Locomotor activity patterns (mean ambulatory and total motor activity counts) were unaffected by test substance administration.

Sacrifice and pathology:

Gross pathology: There were no macroscopic changes observed for the 5 animals/sex in the test substance-exposed groups that were perfused. A single finding of red material was noted in the brain for 1 control group male, but was considered a spontaneous, incidental finding, which had no correlating microscopic findings.

Brain weight: Group mean absolute brain weight for males was statistically significantly lower than the concurrent control at all dose levels (↓8-11%). While the differences from the concurrent control group for the 50 and 500 ppm group males were statistically significant, on an individual basis, all brain weights from the perfused males in these groups (except 1 male in the 500 ppm group) were within the mean range of the WIL historical control database. In contrast, 4 of the 5 brain weights from perfused concurrent control group males were higher than the maximum mean value in the WIL historical control database. Therefore, the differences in mean brain weight for the 50 and 500 ppm group males were attributed to a high concurrent control group mean and were considered not to be test substance-related. Mean brain weight for the 5000 ppm group males (5 males examined) was 11.3% lower (statistically significant) than the control group. The relationship to treatment of the apparently lower group mean brain weight, although equivocal, cannot conclusively be discounted and is therefore considered test substance-related. In light of the absence of any effect on brain weight in females and the absence of any corroborating effects on FOB or neuropathological findings, this difference is considered likely not to be of toxicological significance. Data are presented in table 6.

Table 6: Intergroup comparison of absolute brain weights

	Dose level of bicyclopyrone (ppm)							
	Males				Females			
	0	50	500	5000	0	50	500	5000
Absolute Mean Brain Weights	2.38 ± 0.112	2.20* ± 0.078 (↓8%)	2.20* ± 0.095 (↓8%)	2.11** ± 0.105 (↓11%)	2.06 ± 0.099	2.03 ± 0.072	2.00 ± 0.163	1.99 ± 0.053

Data were taken from pages 220-221 of the study report
*statistically significantly different from control at 0.05 (Dunnett's test)
**statistically significantly different from control at 0.01 (Dunnett's test)

Neuropathology: There were no bicyclopyrone-related histologic changes. All histologic changes were considered to be incidental findings or related to some aspect of experimental manipulation other than administration of bicyclopyrone. There was no bicyclopyrone-related alteration in the prevalence, severity, or histologic character of those incidental tissue alterations.

INVESTIGATOR'S CONCLUSIONS:

The NOEL for subchronic neurotoxicity was considered to be 500 ppm for males (35 mg/kg/day) based on a lower group mean brain weight at 5000 ppm, for which a relationship to treatment cannot be excluded, and 5000 ppm for females (415 mg/kg/day) when bicyclopyrone was offered in the diet for 90 days.

REVIEWER'S COMMENTS:

Canada (PMRA) was of the opinion that language about the mode of action of bicyclopyrone should be put into ophthalmoscopic observations section. While EPA agrees that these effects are indicative of a similar mode of action to other chemicals within its class, the agencies disagree about placing this language in the results section. EPA, PMRA and APVMA all agree that the decreased brain weights are not treatment related and adverse based upon; 1) a lack of findings in the various functional tests; 2) no histopathological findings in the selected neural tissues; 3) lack of evidence of neurotoxic potential in other studies throughout the bicyclopyrone database 4) the changes were in the historical control range.

Based upon the effects of this study, the systemic LOAEL is 50 ppm (4 mg/kg/day) based upon an increased incidence of unilateral keratitis in the eyes of males. The systemic NOAEL was not observed.

Neurotoxicity NOAEL = 5000 ppm (336/415 mg/kg/day [M/F]). Neurotoxicity LOAEL was not observed.

This study is classified as totally reliable (**acceptable/guideline**) and satisfies the guideline requirements (OPPTS 870.6200; OECD 424) for a subchronic neurotoxicity screening study in rats. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Beck M. 2012)

Appendix

STUDY TYPE: Range-Finding Subchronic Neurotoxicity Study in Rodents OECD 424 (1997): OPPTS 870.6200b (1998): 2004/73/EC B.43 (2004)

TEST MATERIAL (PURITY): NOA449280 (94.5 % w/w)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Beck, M., 2012. A 28-Day Preliminary Study of bicyclopyrone in Rats. WIL Research Laboratories, LLC, 1407 George Road, Ashland, OH 44805-8946, USA. Laboratory Report No. WIL-639016, issue date 23 April 2012 Unpublished. (Syngenta File No. NOA449280_11146)

SPONSOR: Syngenta, Ltd. Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, United Kingdom

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In a 28-day preliminary subchronic neurotoxicity study, bicyclopyrone (NOA449280, 94.5 % w/w), was offered on a continuous basis in the diet for 28 days to 3 groups (Groups 2-4) of Crl:CD (SD) rats at target concentrations of 500, 2500 and 5000 ppm (50/53, 240/259 and 471/505 mg/kg/day [M/F]). A concurrent control group (Group 1) was provided the basal diet on a comparable regimen. Each group consisted of 8 males and 8 females. Animals were approximately 6 weeks old at the initiation of dose administration. Detailed physical examinations were performed weekly. Individual body weights and food consumption were recorded weekly. All animals were euthanized on study day 28, and a gross necropsy was performed.

At 500 ppm bicyclopyrone, there were no treatment-related findings.

At 2500 ppm bicyclopyrone, males exhibited lower mean body weight gains than control animals during the first week of treatment (↓16%). There was no change in absolute body weights. There were minor changes in mean food efficiencies during study week 0 to 1 which correlated to the decreased body weight gains and food consumption.

At 5000 ppm bicyclopyrone, lower mean body weight gains were noted for the 5000 ppm males and females after the first week of treatment (↓21 and ↓23%). There was no change in absolute body weights. The males in the 5000 ppm group had corresponding decreased food consumption (↓12%). There were minor changes in mean food efficiencies during study week 0 to 1 which correlated to the decreased body weight gains and food consumption.

Based upon the results of this study the NOAEL for systemic and neurotoxicity is 5000 ppm (471/505 mg/kg/day [M/F]). A LOAEL was not observed.

This study is classified as reliable (**acceptable/non-guideline**) and satisfies the guideline requirements (OPPTS 870.6200b; OECD 408) for a subchronic oral neurotoxicity range-finding toxicity study in the rat.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

Primary Reviewer: Monique M. Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 3/18/15
Secondary Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar J. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 03/18/15

ABBREVIATED DATA EVALUATION RECORD

TXR NO: 0057111

STUDY TYPE: Metabolism - rat; OPPTS 870.7485 [§85-1]; OECD 417; 87/302/EEC B.36 (1987) 94/79/EC (1994); JMAFF 12 Nohsan No 8147 (2000)

PC CODE: 018986 **DP BARCODE:** D425155

TEST MATERIAL (PURITY): NOA449280 (99.9%)

SYNONYMS: Bicyclopyrone; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one; Bicyclo[3.2.1]oct-3-en-2-one, 4-hydroxy-3-[[2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]

CITATION: Hurst, L. (2009). [14C]-NOA449280 – Excretion and tissue distribution following single oral and intravenous administration to the rats. Covance Laboratories Ltd., North Yorkshire, UK. December 23, 2009. Study Number: 1983/094. MRID #47841961. Unpublished.

SPONSOR: Syngenta Limited, Bracknell, Berkshire, UK.

EXECUTIVE SUMMARY:

In a tissue and excretion study (MRID# 47841961), radiolabeled bicyclopyrone [pyridinyl-3-^{14}C]-NOA449280 (99.9% purity, batch #AMS 1144/1) was administered to 4 Crl:WI(Han) rats/sex/group as a single oral gavage dose of 2 or 200 mg/kg or a single intravenous (IV) dose at 2 mg/kg. Urine, feces, and cage washes were collected at pre-determined intervals over 7 days then analysed for radioactivity content. Rats were then sacrificed and residual radioactivity was measured in blood and plasma, selected tissues, and the remaining carcass.

The major routes of excretion after a single oral dose of 2 mg/kg were the urine (64% and 85% of the administered dose in males and females, respectively) and feces (27% and 5% of the administered dose in males and females, respectively). The majority of the administered dose was eliminated within 24 hours of dosing. No radioactivity was detected in expired air. Small amounts of radioactivity were detected in the cage wash after 7 days (3% and 1% of the administered dose in males and females, respectively). Radioactive residues after 7 days were low and the tissue distribution was similar between sexes. The highest levels of radioactivity were found in the liver (4% and 3% of the administered dose in males and females, respectively) and kidney (0.3% and 0.4% of the administered dose in males and females, respectively). Mean total recoveries in these groups were within acceptable limits (100% and 99% of the

administered dose in males and females, respectively).

Administration of a single oral dose of 200 mg/kg resulted in similar results as those obtained for the low dose. The major routes of excretion were the urine (68% and 88% of the administered dose in males and females, respectively) and feces (23% and 6% of the administered dose in males and females, respectively). Small amounts of radioactivity were detected in the cage wash after 7 days (5% and 4% of the administered dose in males and females, respectively). The majority of the administered dose was eliminated within 24 hours of dosing and no radioactivity was detected in the expired air. Radioactive residues after 7 days were low with the highest levels of radioactivity found in the liver (0.1% of the administered dose in both sexes). Mean total recoveries in these groups were within acceptable limits (97% and 98% of the administered dose in males and females, respectively).

Administration of a single intravenous dose at 2 mg/kg resulted in similar results as those obtained for a single oral dose at 2 mg/kg. The major routes of excretion were the urine (63% and 87% of the administered dose for males and females, respectively) and feces (29% and 5% of the administered dose for males and females, respectively). Small amounts of radioactivity were detected in the cage wash after 7 days (up to 5% of the administered dose). The majority of the administered dose was eliminated within 24 hours of dosing and no radioactivity was detected in the expired air. Radioactive residues after 7 days were low with highest levels of radioactivity in the liver (4–5% of the administered dose in both sexes) and kidney (0.3% and 0.5% of the administered dose in males and females, respectively). Mean total recoveries in these groups were within acceptable limits (100% and 102% of the administered dose in males and females, respectively).

The mean percentage recovered radioactivity was similar across the three dosing schemes, within each sex. Compared to males, females excreted higher proportions in the urine. Overall, these data indicate rapid and almost complete absorption of bicyclopyrone, with the majority of the administered dose excreted via the urine within 24 hours.

CLASSIFICATION:

This metabolism study in the rat is classified as totally reliable **(acceptable/non-guideline)**. In combination with the other submitted ADME studies (MRID#s 47841962, 47841963, 47841964 and 47842110), it satisfies the guideline requirement for a metabolism study [OPPTS 870.7485, OECD 417] in rats. EPA, PMRA (Canada), and APVMA/OCS agree on the regulatory decision and classification for this study.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

EPA Reviewer: Anwar Y. Dunbar, Ph.D. **Signature:** Anw Y. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 03/19/15
EPA Secondary Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 3/19/15

ABBREVIATED DATA EVALUATION RECORD

TXR#: 0057111

STUDY TYPE: Metabolism – rat ; OPPTS 870.7485 [§85-1]; OECD 417; 87/302/EEC B.36 (1987) 94/79/EC (1994); JMAFF 12 Nohsan No 8147.

PC CODE: 018986 **DP BARCODE:** D425155

TEST MATERIAL (PURITY): Bicyclopyrone (99.9%)

SYNONYMS: NOA449280, 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-trifluoromethyl-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one, Bicyclo[3.2.1]oct-3-en-2-one, 4-hydroxy-3-[[2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]

CITATION: Hardwick T and Swalwell L Author (31 May 2013) [14C]-NOA449280 - Biotransformation in the Rat. Covance Laboratories Limited (Covance Laboratories Limited, Otley Road, Harrogate, North Yorkshire, HG3 1PY, UK). Report Number: 1983/098, Study Number: 1983/098, Task Number: T013086-05. MRID #47841962. Unpublished.

SPONSOR: Syngenta Limited, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK

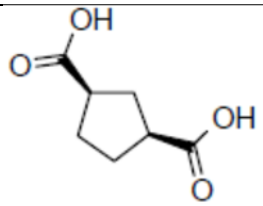
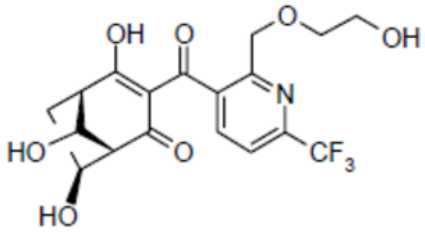
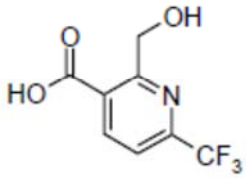
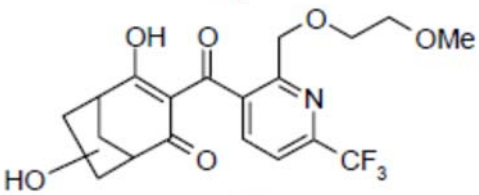
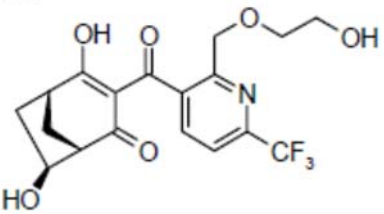
EXECUTIVE SUMMARY:

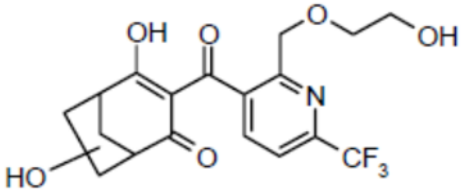
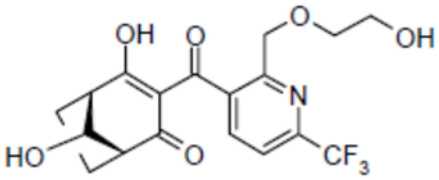
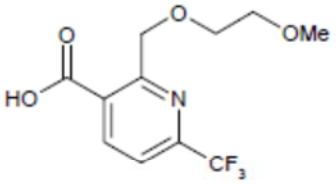
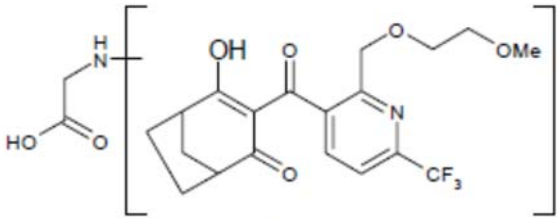
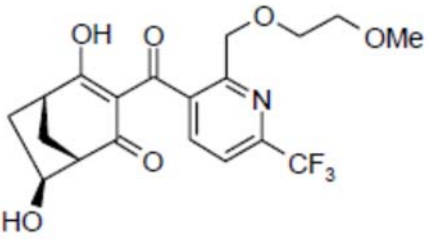
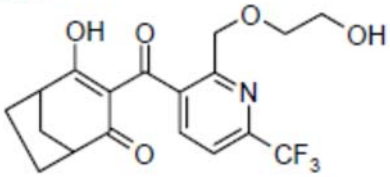
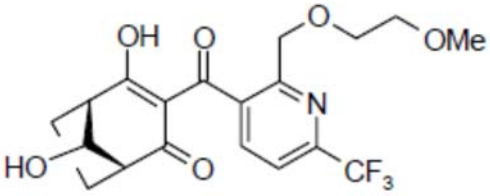
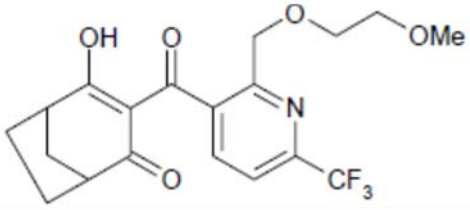
In a metabolism study (MRID# 47842110) Bicyclopyrone (99.9%a.i., batch/lot # - AMS 1144/1 (CHD number 0435/07-1983 Lot 4), [pyridinyl-3-^{14}C]-NOA449280] was administered to 2-4 Han Wistar rats per sex in 0.1M sodium carbonate by gavage at dose levels of 2 or 200 mg/kg (single and repeat dose), or a single intravenous dose (2 mg/kg). Plasma and excreta from toxicokinetic studies with bicyclopyrone (Study Numbers: 1983/093, 1983/094, 0983/078, 1983/103 and 1983/111) were analysed (see following table). Quantitative metabolic profiles were determined by radio-chromatography and HPLC-MS.

Study No.	Dose and route	Matrix	Time point (hours)	Sex	Group size
1983/093	2 mg/kg oral	Plasma	1	Male and female	2/sex
	200 mg/kg oral	Plasma	2	Male and female	2/sex
	2 mg/kg iv	Plasma	0.083	Male and female	2/sex
1983/094	2 mg/kg oral	Urine	0-48	Male and female	4/sex
	2 mg/kg oral	Feces	0-48	Male and female	4/sex
	2 mg/kg oral	Cage wash	0-24	Male and female	4/sex
	200 mg/kg oral	Urine	0-48	Male and female	4/sex

	200 mg/kg oral	Feces	0-72	Male and female	4/sex
	200 mg/kg oral	Cage wash	0-24	Male and female	4/sex
1983/078	2 mg/kg oral	Urine	0-48	Male and female	
	2 mg/kg oral	Feces	0-48	Male and female	
	2 mg/kg oral	Cage wash	0-24	Male and female	
	2 mg/kg oral	Bile	0-48	Male	
	2 mg/kg oral	Bile	0-24	Female	
	200 mg/kg oral	Urine	0-48	Male and female	
	200 mg/kg oral	Feces	0-48	Male and female	
	200 mg/kg oral	Cage wash	0-24	Male and female	
	200 mg/kg oral	Bile	0-48	Male	
	200 mg/kg oral	Bile	0-24	Female	
1983/103	2 mg/kg oral	Urine	28 days after the	Male	
	2 mg/kg oral	Feces	final dose	Male	
1983/111	2 mg/kg oral	Liver	6	Male and female	
	200 mg/kg oral	Liver	6	Male and female	

The following compounds/metabolites were identified:

Metabolite	Structure
CSAA589691 (NOA412101)	
CSCD677692	
CSAA806573	
Hydroxy NOA449280	
CSCD675162	

Metabolite	Structure
Desmethyl monohydroxy NOA449280 (2 isomers)	
CSCD677693	
SYN503780 (CSAA794148)	
NOA449280 (Glycine)	
CSCD675164	
CSAA915194 (NOA454598)	
CSCD677306	
NOA449280 (parent compound; CSAA798499)	

Bicyclopyrone (NOA449280) was not extensively metabolized; the unchanged parent was the

principle radiolabelled component in all dose groups. The principal biotransformation routes were *via* oxidative phase 1 reactions.

At both dose levels, the major radioactive component in the urine of non-cannulated rats was parent compound, accounting for *ca* 40 and 80% of the dose in males and females respectively. The major urinary metabolite was the O-demethylated metabolite CSAA915194 accounting for *ca* 10 and 2% of the dose in males and females respectively. Minor metabolites included CSCD675164 (5/1% at low dose, 8/2% at high dose; M/F) and CSCD677306 (3/0.7% at low dose, 4/1% at high dose; M/F) and, detected in males only, CSCD675162 (4% at low dose, 3% at high dose) and CSCD677693 (1% at both dose levels). The major radioactive component in cage wash was parent compound accounting for 2 to 5% of the administered dose which was considered to originate from urinary contamination of the metabolism cage.

The major fecal metabolite was the monohydroxylated metabolite CSCD675164 accounting for *ca* 11 and 2% of the dose at the low dose level and for *ca* 8 and 0.4% at the high dose level in males and females respectively. Minor metabolites included CSCD675162 (2–3% in males at both dose levels) and CSCD677306 (3% in males, 0.5–1% in females at both dose levels). Parent compound accounted for less than 3% of the dose. The metabolite profile obtained from rats administered multiple low doses of bicyclopyrone was similar to that obtained from the equivalent single dose.

In bile duct cannulated rats, the metabolite profile in urine was similar to that obtained from intact rats. The major biliary metabolite was the monohydroxylated metabolite CSCD675164 accounting for *ca* 7 and 1% of the dose at the low dose level and for *ca* 5 and 1% at the high dose level in males and females respectively. Minor metabolites included CSAA915194 (2–3% for males, 0.1–0.3% for females) and CSCD677306 (1–2% for males, 0.2–0.3% for females). Parent compound accounted for *ca* 2% of the dose at the low dose level and for 10 and 4% of the dose at the high dose level for males and females respectively. The major radioactive component in the faeces of cannulated rats was parent compound (3–10%).

The major circulating component in plasma was parent compound. Minor metabolites included CSAA915194, CSCD677692 and CSCD675164.

The major component in liver was parent compound. Minor metabolites included CSAA915194 and CSCD677306.

The biotransformation of [^{14}C]-NOA449280 was qualitatively independent of dose level and dose route, however, there was a quantitative sex difference in the metabolism with males metabolising a higher proportion of parent compound than females. Repeated administration had no significant effect on the metabolic profile, indicating no induction or inhibition of metabolic enzymes. Unchanged parent compound was the principle component in all dose groups. The most prevalent route of metabolism was by hydroxylation and O-demethylation. Minor routes involved glycine conjugation and cleavage between the pyridinyl and bicyclo rings (each accounting for less than 0.5% of the dose). There was a quantitative sex difference apparent in the metabolism of bicyclopyrone with males transforming a higher proportion of parent compound into metabolites than females.

All metabolites accounting for >5% of the dose and the majority of metabolites accounting for

>1% of the dose were identified. Therefore, greater than 90% of the excreted dose, equivalent to 74-96% of the administered dose was accounted for by identified metabolites and parent compound.

CLASSIFICATION:

This metabolism study in the rat is classified as **acceptable/non-guideline**. In combination with the other submitted ADME studies (MRID#s 47841961, 47841963, 47841964 and 47842110), it satisfies the guideline requirement for a metabolism study [OPPTS 870.7485, OECD 417] in rats. EPA, PMRA (Canada), and APVMA/OCS agree on the regulatory decision and classification for this study.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

Primary Reviewer: Monique M. Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 3/18/15
Secondary Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anw J. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 03/18/15

ABBREVIATED DATA EVALUATION RECORD

TXR NO: 0057111

STUDY TYPE: Metabolism - rat; OPPTS 870.7485 [§85-1]; OECD 417; 87/302/EEC B.36 (1987) 94/79/EC (1994); JMAFF 12 Nohsan No 8147.

PC CODE: 018986 **DP BARCODE:** D425155

TEST MATERIAL (PURITY): NOA449280 (99.9%)

SYNONYMS: Bicyclopyrone; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one; Bicyclo[3.2.1]oct-3-en-2-one, 4-hydroxy-3-[[2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]

CITATION: Hurst, L. and Stow, R.(2010). [14C]-NOA449280 – Tissue depletion in the rat following a single oral administration. Covance Laboratories Ltd., North Yorkshire, UK. February 3, 2010. Study Number: 1983/111. MRID# 47841963. Unpublished.

SPONSOR: Syngenta Limited, Bracknell, Berkshire, UK.

EXECUTIVE SUMMARY:

In a tissue depletion study (MRID 47841963), radiolabeled bycyclopyrone [pyridinyl-3-^{14}C]-NOA449280 (99.9% purity, batch #AMS 1144/1) was administered to Crl:WI(Han) as a single oral gavage dose at 2 or 200 mg/kg (21/sex/dose). At 2, 6, 12, 24, 48, 96 and 144 hours post dose administration, animals were sacrificed (3/sex/dose/observation point) and radioactivity measured in selected tissues and the residual carcass. Elimination half-lives were calculated for all tissues where concentrations were detected at more than three time points and where a terminal elimination phase could be unambiguously defined. Excreta were not collected.

At 2 mg/kg, the mean maximum levels of radioactivity (C_{max}) were measured at the first sampling time (2 hours post dosing) for all tissues except the GI tract and contents for males only. Levels of total radioactivity were detectable in all tissues investigated at 2 hours post dose with the highest concentrations seen in the liver (8.1 and 6.3 µg equivalents/g in males and females, respectively), GI tract and contents (7.3 and 4.0 µg equivalents/g in males and females, respectively), and kidney (4.4 and 3.5 µg equivalents/g in males and females, respectively). Notable radioactivity was also detected in the blood (1.6 and 1.1 µg equivalents/g in males and females, respectively) and plasma (2.5 and 1.7 µg equivalents/g in males and females, respectively). Total radioactivity in tissues declined rapidly with $C_{max}/2$ achieved within 6 hours after dosing and approaching the limit of quantification for the majority of tissues 24 hours after dosing, with the exception of the liver, kidneys, and GI tract and contents. At 144 hours post

dosing, the liver and kidney contained the highest concentrations (2.1 and 2.0 µg equivalents/g in male and female liver respectively and 0.7 and 1.3 µg equivalents/g in male and female kidney respectively). All other tissues were approaching or below the limit of detection. Radioactivity in the carcass accounted for approximately 0.4% and 0.7% of the administered dose for males and females, respectively. Calculated elimination half-lives in tissues were 3.9-191.5 hours in males and 1.4-15.3 hours in females (Table 1). The longest estimated half-life was for renal fat and the uterus, for male and female rats, respectively.

At 200 mg/kg, C_{max} in selected tissues was measured at 2 hours post dosing with the exception of the renal fat (males only) and kidneys (both sexes) where C_{max} was observed at 6 hours post dosing. Radioactivity was detected in all tissues investigated at this time point with the highest concentrations seen in the GI tract and contents (1070 and 1154 µg equivalents/g in males and females respectively), plasma (301.2 and 206.6 µg equivalents/g in males and females respectively), blood (210.6 and 147.4 µg equivalents/g in males and females respectively) and liver (202.4 and 176.8 µg equivalents/g in males and females respectively). Total radioactivity in tissues declined rapidly with $C_{max}/2$ achieved between 6 and 12 hours after dosing and approaching the limit of quantification for the majority of tissues 48 hours after dosing. Distribution of radioactivity was similar to that observed at 2 hours post dosing. At 144 hours post dosing, the highest concentrations were observed in the liver (5.1 and 3.9 µg equivalents/g in males and females, respectively) and kidneys (1.7 and 2.2 µg equivalents/g in males and females, respectively) with additional radioactivity detected at <1.7 µg equivalents/g in the spleen, thymus, heart, lung, and muscle in both sexes, and pancreas and uterus in females only. Radioactivity in the carcass accounted for approximately 0.3% and 0.2% of the administered dose for males and females, respectively. Calculated elimination half-lives in tissues were 2.3-51.2 hours in males and 1.7-481 hours in females (Table 2). The longest estimated half-life was for muscle and kidneys, for male and female rats, respectively.

Table 1. Calculated Elimination Half-Lives of Radioactivity in Tissues Following a Single Oral Administration of 2 mg/kg.

	Males		Females	
Tissue type	T½ (hrs)	R^2	T½ (hrs)	R^2
Adrenals	82.06	0.954	NR	NR
Blood	NR	NR	1.413	0.916
Bone	NC	NA	NC	NA
Brain	3.928	0.983	NR	NR
Fat (Renal)	191.5	0.964	NR	NR
G.I. Tract	NR	NR	NR	NR
Heart	NR	NR	NR	NR
Kidney	NR	NR	NR	NR
Liver	NR	NR	NR	NR
Lung	NR	NR	NR	NR
Muscle	78.08	0.931	NR	NR
Ovaries	NA	NA	1.524	0.915
Pancreas	NR	NR	13.61	0.987
Plasma	60.57	0.978	NR	NR
Spleen	NR	NR	NR	NR
Testes	NR	NR	NA	NA

Thymus	NR	NR	NR	NR
Thyroid	NC	NA	NR	NR
Uterus	NA	NA	15.29	0.995

NC – Not Calculated, insufficient data points occurred in the elimination phase to accurately determine a tissue elimination half life.
NR – Not reported; correlation coefficient <0.9 for elimination phase
NA – Not applicable

Table 2. Elimination Half-Lives of Radioactivity in Tissues Following a Single Oral Administration of 200 mg/kg.

	Males		Females	
Tissue type	T½ (hrs)	R^2	T½ (hrs)	R^2
Adrenals	2.507	0.996	NR	NR
Blood	2.603	0.999	NR	NR
Bone	2.343	0.998	NR	NR
Brain	3.173	0.992	NR	NR
Fat (Renal)	2.753	0.992	1.744	0.924
G.I. Tract	9.197	0.925	NR	NR
Heart	NR	NR	NR	NR
Kidney	NR	NR	481.1	0.988
Liver	NR	NR	NR	NR
Lung	NR	NR	NR	NR
Muscle	51.23	0.904	NR	NA
Ovaries	NA	NA	NR	NR
Pancreas	NR	NR	NR	NR
Plasma	2.554	0.999	NR	NR
Spleen	NR	NR	NR	NR
Testes	NR	NR	NA	NA
Thymus	NR	NR	NR	NR
Thyroid	3.078	0.918	3.043	0.987
Uterus	NA	NA	NR	NR

NR – Not reported; correlation coefficient <0.9 for elimination phase
NA – Not Applicable

CLASSIFICATION:

The APVMA/OCS (Australia) proposed including data to show the % radioactivity recovered over time in a table, as there would be benefit in showing the excretion in this study is similar to that observed in other studies, i.e., rapid and almost complete. This may be as simple as showing the sub total for the various tissues, carcass and the total % recovery – for each sex and dose – using -the data from pages 70-83. Due to a constraint in resources, this change was not incorporated.

This metabolism study in the rat is classified as **acceptable/non-guideline**. In combination with the other submitted ADME studies (MRID#s 47841961, 47841962, 47841964 and 47842110), it satisfies the guideline requirement for a metabolism study [OPPTS 870.7485, OECD 417] in rats. EPA, PMRA (Canada), and APVMA/OCS agree on the regulatory decision and

classification for this study.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

EPA Reviewer: Anwar Y. Dunbar, Ph.D. **Signature:** Anwar Y. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 03/19/15
EPA Secondary Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 3/19/15

ABBREVIATED DATA EVALUATION RECORD

TXR#: 0057111

STUDY TYPE: Metabolism - rat; OPPTS 870.7485 [§85-1]; OECD 417; 87/302/EEC B.36 (1987) 94/79/EC (1994); JMAFF 12 Nohsan No 8147.

PC CODE: 018986 **DP BARCODE:** D425155

TEST MATERIAL (PURITY): Bicyclopyrone (99.9%)

SYNONYMS: NOA449280, 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-trifluoromethyl-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one, Bicyclo[3.2.1]oct-3-en-2-one, 4-hydroxy-3-[[2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]

CITATION: L Hurst and R Stow (2009) An Investigation into the Pharmacokinetics Following Single Oral and Intravenous Administration to the Rat. Covance Laboratories Limited (Covance Laboratories Limited, Otley Road, Harrogate, North Yorkshire, HG3 1PY, UK), (23 December 2009). Report Number: 1983/093, Study Number: 1983/093, Task Number: T001274-07. MRID# 47841964. Unpublished.

SPONSOR: Syngenta Limited, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK

EXECUTIVE SUMMARY:

In a pharmacokinetic study (MRID# 47841964) Bicyclopyrone [(99.9%a.i., batch/lot # - AMS 1144/1 (CHD number 0435/07-1983 Lot 4), (pyridinyl-3-^{14}C)-NOA449280] was administered to Han Wistar rats (4/sex/dose) in 0.1M sodium carbonate by oral gavage at dose levels of 2 or 200 mg/kg, or a single intravenous (IV) dose at 2 mg/kg. Plasma and blood concentrations were determined pre-dose, and at 0.167, 0.5, 0.75, 1, 2, 4, 8, 12, 24, 48, 72 and 96 hours post-dose (iv groups were measured 0.083 and 0.25 hours but not at 0.167 hours).

Maximum mean plasma concentrations (Cmax) were reached in both male and female rats within 1.3-2.3 hours after oral dosing, indicating rapid absorption into the systemic circulation. Cmax values increased proportionally with administered dose at 3.3 and 2.9 µg equivalents/g for 2 mg/kg bw males and females, respectively, and 425 and 441 µg equivalents/g for 200 mg/kg bw males and females, respectively. The levels of radioactivity in plasma declined rapidly in a biphasic pattern. The mean plasma clearance half life values of the first pbase (α-phase) were estimated at 2.7 and 2.4 hours for 2 mg/kg bw males and females, respectively, and were noted to increase modestly at 200 mg/kg bw to 3.2 hours in males, but not in females (1.8 hours). At the low dose of 2 mg/kg bw, it was not possible to calculate a half-life value for the second phase (β-phase), because the majority of samples were at or below the limit of detection. At 200 mg/kg

bw, the mean plasma β half-life values were estimated at 12.5 and 68.6 hours in males and females respectively.

Following a single intravenous dose of [^{14}C]-NOA449280 at a nominal dose level of 2 mg/kg, the concentration of radioactivity in plasma extrapolated to the zero time point (C_0) was 6.1 and 6.6 µg equivalents/g in male and female rats respectively. Plasma concentrations declined rapidly in a biphasic pattern with mean α half-life values of 2.0 and 1.4 hours for males and females, respectively. A second-phase β half-life value was not determined due to rapid clearance of plasma radiolabel to LOD levels.

At all dose levels and routes, systemic exposure as described by plasma AUC appeared to be slightly higher in males than females. Comparison of the AUC values obtained following a low oral dose with the intravenous dose indicates very similar plasma profiles and an oral bioavailability of approximately 97% for males and 85% in females. The mean values obtained for C_{max} were generally proportional to dose, but AUC values obtained for the high dose appeared to be supra proportional to dose. However, this is considered to be attributable to differences in the calculation of AUC between the two dose levels due to the definition of the β-phase in the high dose level only.

The concentration of radioactivity in blood followed the same pattern as plasma, with plasma concentrations generally slightly higher than those in blood suggesting no preferential uptake into red blood cells in all dose groups analysed.

In summary, following single oral administration of either 2 or 200 mg [^{14}C]-NOA449280/kg, absorption was rapid and quantitative. Plasma concentrations of radioactivity declined rapidly with a half-life ($t_{½}$) of ~2-4 hours (α-phase) and were at or below the limit of detection by 24 hours after dosing. The plasma profile of radioactivity obtained following a single intravenous dose of 2 mg [^{14}C]-NOA449280/kg was similar to the equivalent oral dose. Systemic exposure appeared to be slightly higher for males than for females.

Mean Plasma Pharmacokinetic Parameters Following an Oral or IV Administration of [^{14}C]-NOA449280 to Male and Female Rats

	Oral				IV	
Time point (h)	Male 2 mg/kg	Female 2 mg/kg	Male 200 mg/kg	Female 200 mg/kg	Male 2 mg/kg	Female 2 mg/kg
0	NA	ND	ND	ND	ND	ND
0.083	NA	NA	NA	NA	5.630	6.130
0.167/0.25	1.283	0.425	164.8	292.9	4.845	5.348
0.5	3.252	2.360	255.3	366.9	4.355	4.920
0.75	3.307	2.614	274.3	373.6	NA	NA
1	3.192	1.246	295.4	421.3	3.537	3.115
2	2.830	1.717	413.0	380.5	2.175	0.877
4	1.440	1.325	328.3	275.6	1.041	0.587
8	0.248	0.046	111.6	35.63	0.198	0.047
12	0.092	0.036	57.93	10.86	0.077	0.020
24	0.007	0.002	4.853	0.562	0.009	0.009
48	0.004	0.005	0.896	0.343	0.006	0.012
72	ND	ND	0.335	0.346	0.001	0.002
96	0.006	0.003	0.462	0.194	0.005	0.002
C_{max}/C_0 (µg equivalents/g)	3.327	2.929	425.4	440.6	6.078	6.565

T_{max} (h)	1.40	1.30	2.30	1.30	NA	NA
$t_{½\alpha}$ (h)	2.743	2.447	3.197	1.833	1.964	1.421
$t_{½\beta}$ (h)	NC	NC	12.45	68.59	NC	NC
AUC_{0-t} (µg equivalents.h/g)	13.21	8.375	2771	1990	13.68	9.928
Total $AUC_{0-\infty}$ (µg equivalents.h /g)	13.25	8.413	2772	1991	13.70	9.950
Bioavailability* (%)	96.72	84.55	NA	NA	NA	NA

ND – Not Detected
NA – Not Applicable
NC – Not Calculable due to insufficient data points
*Bioavailability calculated as the proportion of oral to iv AUC values for the 2 mg/kg groups
Table was taken from pages 40-41 and 44 of the study report

CLASSIFICATION:

The APVMA/OCS (Australia) proposed inclusion of the I.V. data into the tables in this DER. This change was considered but not incorporated due to time constraints and also the fact it would not impact the risk assessment for bicyclopyrone.

This pharmacokinetic study in the rat is classified as **acceptable/non-guideline**. In combination with the other submitted ADME studies (MRID#s 47841961, 47841962, 47841963 and 47842110), it satisfies the guideline requirement for a metabolism study [OPPTS 870.7485, OECD 417] in rats. EPA, PMRA (Canada), and APVMA/OCS agree on the regulatory decision and classification for this study.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

Primary Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar J. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 03/18/15
Secondary Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 3/18/15

ABBREVIATED DATA EVALUATION RECORD

TXR NO: 005711

STUDY TYPE: Metabolism - rat; OPPTS 870.7485 [§85-1]; OECD 417.

PC CODE: 018986 **DP BARCODE:** D425155

TEST MATERIAL (PURITY): NOA449280 (99.9%)

SYNONYMS: Bicyclopyrone; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one; Bicyclo[3.2.1]oct-3-en-2-one, 4-hydroxy-3-[[2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]

CITATION: Kendrick, J. and Goodwin, D.(2010). [14C]-NOA449280 – Excretion and Tissue Distribution Following Repeated Oral Administration to the Rat . Covance Laboratories Ltd., North Yorkshire, UK. February 3, 2010. MRID# 47841965. Unpublished.

SPONSOR: Syngenta Limited, Bracknell, Berkshire, UK.

EXECUTIVE SUMMARY:

In a tissue depletion study (MRID 47841965), radiolabeled bicyclopyrone [pyridinyl-3-^{14}C]-NOA449280 (99.9% purity, batch #AMS 1144/1) was administered to nine groups of 3 Crl:WI(Han) rats as a single oral gavage dose at 2 mg/kg for 28 days. At prescribed intervals following dose administration, animals were sacrificed and radioactivity was measured in selected tissues and the residual carcass. Elimination half-lives were calculated for all tissues where concentrations were detected at more than three time points and where a terminal elimination phase could be unambiguously defined

The greatest mean concentrations of radioactivity were present in the liver at all time points, containing between 3.6 and 4.3 µg equivalents/g (accounting for up to 1% of the administered dose). The kidney contained the next highest concentration of radioactivity (between 0.9 and 1.0 µg equivalents/g). The mean concentrations of radioactivity in all other tissues were <0.1 µg equivalents/g (≤0.001% of the administered dose).

Mean concentrations of total radioactivity were approximately equally distributed between whole blood and plasma at the time points investigated.

Mean concentrations of radioactivity in the liver and kidney declined following cessation of dosing with elimination half lives of 599 and 1300 hours respectively. Radioactivity concentrations in all other tissues apart from kidney were at least 30 fold lower than in liver, 24 hours after the cessation of dosing, accounting for ≤ 0.001% of the dose during the remainder of the study. See table 1.

The excretion profiles were similar following both the single and repeated oral dosing regimes, with renal elimination accounting for >70% of the administered dose, fecal excretion between 6 and 11%, and cage washes accounting for <5% of the dose administered. See table 2.

Following repeated oral administration of [^{14}C]-bicyclopyrone to male rats at 2mg/kg/day for 28 days, steady-state tissue concentrations were apparently reached by the 10th dose. See tables 3-6.

The highest concentrations of radioactivity were found in the liver and kidney; the concentration of radioactivity in all other tissues examined accounted for ≤0.001% of the dose. There was no evidence of accumulation of radioactivity in any of the tissues examined.

There was no difference in the excretion pattern after single or repeated oral administration of [^{14}C]-bicyclopyrone.

CLASSIFICATION:

This metabolism study in the rat is classified as **acceptable/non-guideline**. In combination with the other submitted ADME studies (MRID#s 47841961, 47841962, 47841963, 47841964 and 47842110), it satisfies the guideline requirement for a metabolism study [OPPTS 870.7485, OECD 417] in rats. EPA, PMRA (Canada), and APVMA/OCS agree on the regulatory decision and classification for this study.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

Table 1. Elimination Half Lives of Radioactivity in Tissues Following 28 Repeated Daily Oral Administrations of [^{14}C]-bicyclopyrone at a Nominal Dose Level of 2 mg/kg Body Weight

Tissue type	$T_{½}$ (hrs)	R^2
Adrenals	NC	NC
Blood	256.5	0.955
Bone	ND	ND
Brain	NC	NC
Fat (Renal)	ND	ND
G.I. Tract	NC	NC
Heart	NC	NC
Kidney	1300	1.000
Liver	598.6	0.993
Lung	NC	NC
Muscle	NC	NC
Pancreas	NC	NC
Plasma	NC	NC
Spleen	NC	NC
Testes	NC	NC
Thymus	NC	NC
Thyroid	ND	ND

ND – Not Determined; insufficient data points occurred in the elimination phase to accurately determine a tissue elimination half life.
NC – Not calculated; correlation coefficient <0.9 for elimination phase
Data were taken from page 42 of the study report.

Table 2. Recovery of Radioactivity in Excreta from Male Rats 24 h After a Single or 24 h After 28 Repeated Oral Administrations of [^{14}C]-NOA449280 at a Nominal Dose Level of 2 mg/kg Body Weight (Group I)

		Recovery of radioactivity (% dose)				
Sample	Number of doses	901M	902M	903M	Mean	SD
Urine	1	63.44	73.17	77.22	71.28	7.080
Urine	28	66.49	73.23	79.01	72.91	6.266
Faeces	1	6.240	5.690	6.916	6.282	0.614
Faeces	28	11.05	11.81	9.750	10.87	1.044
Cage Wash	1	1.206	4.120	2.347	2.558	1.468
Cage Wash	28	3.662	7.298	3.776	4.912	2.067

Data were taken from page 43 of the study report.

Table 3. Concentrations of Radioactivity in Tissues from Male Rats 24 h after 7 Repeated Oral Administrations of [^{14}C]-bicyclopyrone at a Nominal Dose Level of 2 mg/kg Body Weight (Group A)

	Concentration (µg equivalents/g)				
Sample	101M	102M	103M	Mean	SD
Adrenals	0.017	0.038	0.017	0.024	0.012
Blood	0.026	0.020	0.021	0.022	0.003
Bone	ND	0.011	ND	0.003	0.006
Brain	0.025	0.016	0.020	0.020	0.004
Fat (Renal)	0.011	ND	ND	0.003	0.006
Heart	0.040	0.045	0.049	0.045	0.004
Kidney	1.008	0.859	1.016	0.961	0.088
Liver	3.381	3.701	3.890	3.657	0.257
Lung	0.046	0.060	0.051	0.052	0.007
Muscle	0.017	ND	0.017	0.011	0.010
Pancreas	0.195	0.033	0.049	0.092	0.089
Plasma	0.032	0.024	0.022	0.026	0.005
Spleen	0.097	0.078	0.076	0.084	0.011
Testes	0.020	0.013	0.014	0.016	0.003
Thymus	0.061	0.047	0.050	0.053	0.007
Thyroid	ND	ND	ND	ND	NA

ND: Not Detected (taken as zero in calculation of the mean. As such, mean values may be calculated from values above and below the LOD)
NA: Not Applicable
Data were taken from page 31 of the study report.

Table 4. Concentrations of Radioactivity in Tissues from Male Rats 24 h after 10 Repeated Oral Administrations of [^{14}C]-bicyclopyrone at a Nominal Dose Level of 2 mg/kg Body Weight (Group B)

	Concentration (µg equivalents/g)				
Sample	201M	202M	203M	Mean	SD
Adrenals	ND	ND	0.034	0.011	0.019
Blood	0.018	0.014	0.014	0.015	0.001
Bone	ND	ND	ND	ND	NA
Brain	0.028	ND	0.017	0.015	0.014
Fat (Renal)	ND	ND	ND	ND	NA
Heart	0.059	0.034	0.030	0.041	0.015
Kidney	0.911	0.897	0.893	0.900	0.009
Liver	4.333	3.797	3.723	3.951	0.332
Lung	0.046	0.054	0.046	0.049	0.004
Muscle	0.015	0.016	0.013	0.015	0.001
Pancreas	0.042	0.047	0.061	0.050	0.009
Plasma	0.021	0.014	0.013	0.016	0.004
Spleen	0.088	0.034	0.056	0.060	0.027
Testes	0.010	0.012	0.011	0.011	0.001
Thymus	0.048	0.044	0.048	0.046	0.002
Thyroid	ND	ND	ND	ND	NA

ND: Not Detected (taken as zero in calculation of the mean. As such, mean values may be calculated from values above and below the LOD)
NA: Not Applicable
Data were taken from page 32 of the study report.

Table 5. Concentrations of Radioactivity in Tissues from Male Rats 24 h after 21 Repeated Oral Administrations of [^{14}C]-bicyclopyrone at a Nominal Dose Level of 2 mg/kg Body Weight (Group C)

	Concentration (µg equivalents/g)				
Sample	301M	302M	303M	Mean	SD
Adrenals	0.033	ND	ND	0.011	0.019
Blood	0.032	0.026	0.014	0.024	0.009
Bone	ND	ND	ND	ND	NA
Brain	0.018	0.023	0.028	0.023	0.005
Fat (Renal)	ND	ND	ND	ND	NA
Heart	0.063	0.065	0.046	0.058	0.010
Kidney	1.043	0.802	0.976	0.940	0.124
Liver	4.543	4.512	3.842	4.299	0.396
Lung	0.068	0.061	0.061	0.064	0.004
Muscle	0.029	0.022	0.030	0.027	0.004
Pancreas	0.072	0.080	0.054	0.068	0.013
Plasma	0.040	0.025	0.010	0.025	0.014
Spleen	0.091	0.072	0.074	0.079	0.010
Testes	0.021	0.017	0.015	0.017	0.003
Thymus	0.097	0.070	0.067	0.078	0.016
Thyroid	ND	ND	ND	ND	NA

ND: Not Detected (taken as zero in calculation of the mean. As such, mean values may be calculated from values above and below the LOD)
NA: Not Applicable
Data were taken from page 33 of the study report.

Table 6. Concentrations of Radioactivity in Tissues from Male Rats 24 h after 28 Repeated Oral Administrations of [^{14}C]-bicyclopyrone at a Nominal Dose Level of 2 mg/kg Body Weight (Group D)

	Concentration (µg equivalents/g)				
Sample	401M	402M	403M	Mean	SD
Adrenals	ND	0.021	ND	0.007	0.012
Blood	0.017	0.025	0.021	0.021	0.004
Bone	ND	ND	ND	ND	NA
Brain	0.039	0.025	0.031	0.031	0.006
Fat (Renal)	ND	ND	ND	ND	NA
Heart	0.077	0.062	0.077	0.072	0.008
Kidney	1.011	0.834	1.005	0.949	0.100
Liver	3.812	3.682	4.019	3.838	0.170
Lung	0.077	0.064	0.074	0.071	0.006
Muscle	0.033	0.036	0.038	0.036	0.002
Pancreas	0.115	0.123	0.114	0.117	0.004
Plasma	0.012	0.024	0.016	0.017	0.005
Spleen	0.109	0.077	0.108	0.098	0.018
Testes	0.024	0.017	0.017	0.020	0.004
Thymus	0.047	0.105	0.098	0.083	0.031
Thyroid	ND	ND	ND	ND	NA

ND: Not Detected (taken as zero in calculation of the mean. As such, mean values may be calculated from values above and below the LOD)
NA: Not Applicable
Data were taken from page 34 of the study report.

EPA Reviewer: Anwar Y. Dunbar, Ph.D. **Signature:** Anwar Y. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 03/19/15
EPA Secondary Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 3/19/15

ABBREVIATED DATA EVALUATION RECORD

TXR#: 0057111

STUDY TYPE: Metabolism - rat; OPPTS 870.7485 [§85-1]; OECD 417; 87/302/EEC B.36 (1987) 94/79/EC (1994); JMAFF 12 Nohsan No 8147.

PC CODE: 018986 **DP BARCODE:** D425155

TEST MATERIAL (PURITY): Bicyclopyrone (99.9%)

SYNONYMS: NOA449280, 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-trifluoromethyl-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one, Bicyclo[3.2.1]oct-3-en-2-one, 4-hydroxy-3-[[2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]

CITATION: L Hurst Author (16 March 2010) [14C]-NOA449280 - An Investigation into Absorption, Distribution, Metabolism and Biliary Excretion Following a Single Oral Administration to the Rat. Covance Laboratories Limited (Covance Laboratories Limited, Otley Road, Harrogate, North Yorkshire, HG3 1PY, UK). Report Number: 1983/078, Study Number: 1983/078, Task Number: T013085-05. MRID #47842110. Unpublished.

SPONSOR: Syngenta Limited, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK

EXECUTIVE SUMMARY:

In an absorption and excretion study (MRID# 47842110) Bicyclopyrone [(99.9%a.i., batch/lot # - AMS 1144/1 (CHD number 0435/07-1983 Lot 4), [pyridinyl-3-^{14}C]-NOA449280] was administered to bile duct cannulated Han Wistar rats (4/sex/dose, with only 3 rats reported for 2 mg/kg females) in 0.1M sodium carbonate by gavage at dose levels of 2 or 200 mg/kg. Urine, feces, bile and cage washes were collected at pre-determined intervals over 48 hours then analysed for radioactivity content. Rats were then sacrificed and residual radioactivity content was measured in the GI tract and carcass. The absorbed dose was considered to be the percentage of radioactivity recovered in the urine, bile, carcass, and cages washes. The percentage of radioactivity in the cage debris was considered to be unabsorbed, as the origin of this radioactivity is uncertain. The radioactivity identified in the GI tract was considered to be unabsorbed.

Following oral administration at 2 mg/kg it was apparent that renal elimination was the predominant route of excretion with *ca* 42% (males) and *ca* 55% (females) of administered dose recovered in the urine after 48 hours. Males excreted *ca* 14% and *ca* 16% in the feces and bile, respectively, after 48 hours. Females excreted *ca* 5% in the feces and *ca* 2% in the bile,

respectively, after 48 hours. High levels of radioactivity were collected in cage washings with *ca* 19% and *ca* 21% in males and females respectively, which was considered to represent material of urinary origin.

Elimination was rapid since the mean extent of excretion of radioactivity was *ca* 79% (males) and *ca* 70% (females) by 24 hours after dosing, as calculated from the radioactivity recovered in the urine, faeces, bile and cage washings. A mean of *ca* 83% and *ca* 87% of the administered dose was absorbed in males and females, respectively, as calculated from the radioactivity eliminated in urine and bile, together with that present in the residual carcass and cage washings (aqueous and organic). Small amounts of radioactivity were recovered in the GI tract (*ca* 1% for males and 0.4% for females). The mean total percentage recoveries of administered radioactivity including excreta, cage washings, cage debris, GI-tract and residual carcasses were: *ca* 100% for males and *ca* 96% for females.

Following oral administration at 200 mg/kg, urinary elimination accounted for *ca* 69% (females) and *ca* 46% (males) of the total recovered radioactivity after 48 hours. Biliary excretion amounted to *ca* 19% and *ca* 7% in the males and females, respectively, after 48 hours. Fecal excretion accounted for *ca* 8% of administered dose in both sexes, after 48 hours. Approximately 15% (males) and *ca* 13% (females) of the administered dose was recovered in the aqueous and organic cage washings and considered to represent material of urinary origin.

The excretion of radioactivity was rapid with means of *ca* 71% (males) and *ca* 91% (females) of the administered dose recovered 24 hours after dosing, as calculated from the radioactivity recovered in the urine, faeces, bile and cage washings. A mean of *ca* 86% and *ca* 90% of the administered dose was absorbed in males and females, respectively, as calculated from the radioactivity eliminated in urine, associated cage washings (aqueous and organic) and bile, together with that present in the residual carcass. Small amounts of radioactivity were recovered in the GI tract (*ca* 3% for males and 0.3% for females). The total mean percentage recoveries of administered radioactivity including excreta, cage washings, cage debris, GI-tract and residual carcasses were *ca* 98% for both male and female animals.

Following single oral administration of 2 or 200mg [^{14}C]-NOA449280 /kg to bile duct cannulated rats, absorption and elimination was rapid with the majority of the administered dose excreted by the first 24 hours. Renal elimination represented the principal route of excretion of radioactivity independent of sex or dose level, with the majority of the dose being excreted in urine. However, a sex difference was apparent, males excreted a smaller proportion of the administered dose in urine than females and correspondingly more in bile.

Means of 83% and 87% of the low dose were absorbed after 48 hours by males and females respectively. Means of 86% and 90% of the high dose were absorbed after 48 hours by males and females, respectively.

	Absorption after oral administration (Percent of radioactive dose recovered after 48 hours)			
	Group A 2 mg/kg		Group B 200 mg/kg	
	Male (n=4)	Female (n=3)	Male (n=4)	Female (n=4)
Urine	41.68	55.43	45.83	69.41
Bile	16.08	1.838	19.37	7.086
Feces	14.28	5.127	7.622	7.512
Cage wash *	20.56	24.98	15.17	13.02

Carcass	5.022	4.959	6.051	0.486
Cage debris	1.637	3.447	1.408	0.018
GI tract	1.118	0.415	3.01	0.256
% Absorbed	**83.34**	**87.20**	**86.42**	**90.00**
% Total recovery	**100.44**	**99.67**	**98.46**	**97.79**

* - includes; cage wash and final cage wash
Data were taken from pages 32-40 of the study report

CLASSIFICATION:

This absorption and excretion study in the rat is classified as **acceptable/non-guideline**. In combination with the other submitted ADME studies (MRID#s 47841961, 47841962, 47841963 and 47841964), it satisfies the guideline requirement for a metabolism study [OPPTS 870.7485, OECD 417] in rats. EPA, PMRA (Canada), and APVMA/OCS agree on the regulatory decision and classification for this study.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

EPA Reviewer: Anwar Y. Dunbar, Ph.D. **Signature:** Anw Y. Dunbar
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 03/19/15
EPA Secondary Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch 1, Health Effects Division (7509P) **Date:** 3/19/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Rat *in vivo* Dermal Absorption Study OECD 427 (2004): EPA OPPTS 870.7600 (1998)

TEST MATERIAL (PURITY): NOA449280 SL (20% formulation concentrate and two aqueous spray dilutions (1/100 v/v and 1/400 v/v) of the formulation).

SYNONYMS: A16003E.

CITATION: Read H, 2009. NOA449280 SL (A16003E): *In vivo* dermal absorption study in the rat. Quotient Bioresearch (Rushden) Limited, Pegasus Way, Crown Business Park, Rushden, Northamptonshire, NN10 6ER, United Kingdom. Laboratory Report No. SGA/44, 19 November 2009. Unpublished. (MRID 47842239).

SPONSOR: Syngenta Limited, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

In an *in vivo* dermal absorption study (MRID #47842239), [^{14}C]-bicyclopyrone (NOA449280, purity 99.9%), formulated as a 20% soluble liquid (SL) formulation concentrate (A16003E) and as 1/100 and 1/400 aqueous dilutions, was administered to groups of 16 male Han Wistar (Wistar CrL:WI(Han)) rats. Dose application sites of 10 cm^2 per rat were defined and protected by an O-ring glued to the clipped skin surface. Dose preparations of 100 µLwere applied to the skin of each animal corresponding to nominal doses of 20, 0.2 and 0.05 mg bicyclopyrone per rat. Each O-ring was covered with non-occlusive gauze.
Rats were housed individually in metabolism cages for the collection of excreta. After the 6 hour exposure interval, the application sites on all rats were washed with soap and water to remove the unabsorbed dose. Urine, faeces and a cage wash were collected from each cage after the skin wash and at intervals for the duration of each experiment. Subgroups of four rats per dose group were terminated immediately after the 6 hour exposure interval and after 24, 72 and 120 hours after dosing. Cardiac blood samples were collected under terminal anaesthesia. The liver, kidneys and representative samples of adipose tissue and muscle were taken for analysis. Each application skin site was excised and sequentially tape-stripped to remove the *stratum corneum*. The first and second tape strips were transferred into two

separate containers and further tape strips were placed in a third container. All samples, including residual carcasses were analysed for radioactivity. According to agency policy, the potentially absorbable doses from the tape strips (excluding tape strips one and two) were added the absorption estimates.

Following a 6 hour exposure to the formulation concentrate, a mean of 87.3% of the applied radioactivity was unabsorbed and was readily washed from the skin surface using mild soap solution and water. Immediately after the 6 hour exposure interval, approximately 7% of the dose remained associated with the application site; this declined to just 0.8% after 120 hours. The absorbed dose (including potentially absorbable) amounted to 23.49%, 9.58%, 6.33% and 10.04% of the applied dose 6, 24, 72 and 120 hours after dosing, respectively.

Following a 6 hour exposure to the 1/100 aqueous spray dilution, a mean of 82% of the applied radioactivity was unabsorbed and was readily washed from the skin surface. Approximately 15% of the dose remained associated with the application site skin following the 6 hour skin wash; this declined to *circa* 11% after 120 hours. The absorbed dose (including potentially absorbable) amounted to 27.71 %, 14.29%, 14.06% and 17.82% 6, 24, 72 and 120 hours after dosing, respectively.

Following a 6 hour exposure to the 1/400 spray strength dilution, a mean of almost 78% of the applied radioactivity remained unabsorbed and readily washed from the skin surface. Approximately 21.5% of the dose was associated with the application site skin after the 6 hours skin wash; this declined to 9.2% after 120 hours. The dose absorbed, accounted for 24.01%, 20.44%, 17.88% and 19.82% of the applied dose 6, 24, 72 and 120 hours after dosing, respectively.

In all 3 groups, some of the absorbed radioactivity was sequestered into the liver, with lower amounts in the kidney and muscle. The HPPD enzyme which is inhibited by this class of herbicide is located in the liver; hence, the hepatic residues may represent material bound to that enzyme.

Following a 6 hour dermal exposure to bicyclopyrone SL formulation concentrate (A16003E) and to 1/100 and 1/400 spray strength dilutions thereof, most of the applied dose was readily removed from the skin surface by mild skin washing. In all dose groups, residues remained in the skin after washing and thereafter declined in concentration over the 120 hour assessment period, with a corresponding increase in excreta and tissues, indicating continued absorption of the chemical after ceasing exposure.

This study is classified as totally reliable **(acceptable/guideline)** as an *in vivo* dermal absorption study in rats (OPPTS 870.7600).

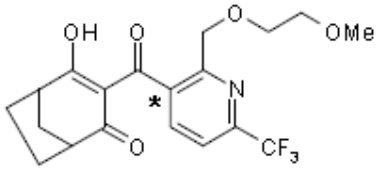
COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, Flagging and Quality Assurance statements were provided.

MATERIALS AND METHODS
Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	White crystalline solid
Lot/Batch number:	AMS 1144/1
Source:	Syngenta Crop Protection
Purity:	99.9%
Stability of test compound:	Confirmed (expiry date 31 May 2011), stored at <30°C.

Structures:

Radiolabelled Test Material:	[Pyridinyl-3-^{14}C]- Bicyclopyrone (NOA449280)
Source::	Syngenta Crop Protection
Batch number:	RDR-V-59
Radiochemical purity:	98.5%
Specific activity:	99.3 µCi/mg (3.6741 MBq/mg)
Stability of test compound:	Confirmed (expiry date 31 August 2009) stored at -20°C.



* denotes the position of [^{14}C]-labelled atoms.

	Blank formulation of A16003E
Blank Formulation:	
Batch number:	J8225/04/1 J8388/065
Source:	Syngenta Ltd.
Storage:	Ambient temperature
Formulation:	NOA449280 200 SL Solo (A16003E)
Source:	Syngenta Ltd.
NOA449280 concentration	Nominally 20% w/v
Storage:	Ambient temperature

Vehicle/Solvent Used:

Name:	CIPAC D water (342 ppm hardness)
Source:	Syngenta Ltd.
Bottle number:	153605
Storage:	Ambient temperature

Relevance of test material to proposed formulation: The formulation concentrate and aqueous dilutions (1/100 and 1/400) were selected to mimic the commercial formulation concentrate and two representative in-use spray strength aqueous dilutions.

Test Animals:

Species:	Rat
Strain:	Han Wistar (Wistar CrL:WI(Han))
Age/weight at dosing:	7-10 weeks / 230-338 g
Source:	Charles River UK Ltd, Margate, Kent, UK
Housing:	Animals group housed in stock cages during acclimatisation. Individually in metabolism cages
Acclimatisation period:	At least 7 days
Diet:	Pellet diet RM1 (E) SQC, (Special Diet Services, Witham, Essex, U.K) *ad libitum*.
Water:	Tap water *ad libitum*
Environmental conditions:	Temperature: 21±2°C Humidity: 45-65% Air changes: Not reported Photoperiod: 12 hours light / 12 hours dark

Study Design and Methods:
Study dates: Start: 26 March 2009 End: 27 July 2009

Dose rationale: The doses and intervals of exposure were selected to represent typical exposures of the commercial formulation concentrate and in-use aqueous spray strength dilutions.

Nominal doses: 20, 0.20 and 0.05 mg ai/10 cm^2 rat skin.

Achieved doses: 19.56, 0.20 and 0.05 mg ai/10 cm^2 skin. The actual doses are the mean of the calculated doses for each rat at each dose level.

Dose volume: 10 µL/cm^2 skin/rat.

Duration of exposures (time from dose to skin wash): 6 hours.

Termination periods (time from dose to sacrifice): 6, 24, 72 and 120 hours.

Number of animals/group: 16 rats per dose level; 4/dose/time point.

Animal preparation: On the day prior to dosing, the fur behind the shoulders of each rat was clipped and the exposed skin swabbed with acetone to remove sebum. The animals were placed in individual glass metabolism cages. On the day of dosing, silicone rubber O-rings (four, previously glued together) were glued to the clipped skin, to define the application site. Care was taken to avoid the inclusion of any damaged skin within each defined area. A non-occlusive nylon gauze was also attached to the dosing device. The total area of skin defined for dose application to each rat was 10 cm^2 per rat.

Dose preparation, administration and quantification:
Preparation: The formulation concentrate was prepared by adding appropriate quantities of [^{14}C]-labelled bicyclopyrone, unlabelled bicycloyrone and the blank formulation. The spray

strength dilutions were prepared by mixing appropriate quantities of [^{14}C]-labelled bicycloyrone, unlabelled bicyclopyrone (1/100 dilution only), blank formulation and water. The details of each dose preparation are presented in the following table.

Table 1: Dose Preparation Details

Study groups	Group 1	Group 2	Group 3
NOA449280 SL (A16003E)	Concentrate	1/100 dilution	1/400 dilution
Dose application volume (µL/10 cm^2/rat)	100	100	100
Nominal dose (mg a.i. per rat)	20	0.20	0.05
Nominal dose (kBq per rat)	500	500	183.7
Unlabelled NOA449280 (mg)	994.698	3.164	0
[^{14}C]-NOA449280 (mg)	6.804	6.804	2.56
Weight of blank formulation added (g)	4.3004	0.043*	0.0129
Weight of water required (g)	0	4.95	5.0123
Weight of dose preparation (g)	5.3068	5.029	5.0252
Specific activity in dose (MBq/mg a.i.)	0.02	2.19	3.38

Specific activity calculations for Group 1 and Group 2 were derived from analysed concentrations of radiolabelled stock solutions. *Weight of blank formulation not recorded, volume measured

Table was taken from page 16 of the study report

Application: The required volume of the formulation concentrate, 1/100 dilution and 1/400 dilution (10 µL/cm^2 and 100 µL per rat) was applied to the defined skin area on each rat, using a positive displacement pipette. The edge of the pipette tip was used to spread the dispensed dose as evenly as practicable over the defined area, taking care to avoid direct contact of the tip or dose preparation with the application site definition device. The pipette tip, used to apply the dose to the application site on each rat, was retained in a container for subsequent solvent extraction of residual radioactivity. A nylon gauze cover protected each application site for the duration of the exposure interval. Immediately after dosing each dosed rat was returned to its metabolism cage.

Quantification / analysis: The radiochemical purity of the [^{14}C]-bicyclopyrone was determined by TLC and HPLC prior to dose preparation and in each dose preparation after dosing. The specific activity and stability of the [^{14}C]-bicyclopyrone in each dose preparation was determined by LSC prior to dosing.

Collection of excreta: Following dosing, urine and faeces were collected from each cage at 6 hours and 24 hours after dosing and then at daily intervals for the duration of each experiment. Excreta and cage washings were stored at approximately -20°C prior to and following analysis. At each sample collection time, each cage was rinsed with a small volume of 1.0M sodium carbonate solution and the wash added to the corresponding urine container.

Skin wash: The application site skin of all rats was washed following the 6 hour exposure interval (the rats terminated at this time were processed as described below). Remaining rats were removed from their cage and held un-anaesthetised over an aluminium foil tray to enable the collection of any excreta. The foil tray was subsequently washed and the washing added to the urine container. The three uppermost O-rings with the attached gauze cover were removed before skin washing (one O-ring remained on the animal). Each application site was washed using nominally 6 pieces of natural sponge pre-wetted with a 3% aqueous solution of Dove liquid and 6 pre-wetted with water, followed by two dry sponges to remove any residual water. Care was taken to avoid the transfer of test substance from the skin surface to the O-

ring with the first few sponges. All of the sponges used for each rat were retained in a single container for [^{14}C]-analysis. The animals were returned to their same metabolism cage. Any urine and faeces excreted during the washing procedure was collected with the respective 0-6h excreta collection. Urine and faeces were collected from each cage immediately after completion of each skin wash.

Termination and Sample Collection: For each dose level, sub-groups of 4 rats were terminated (i) immediately after the 6 hour exposure, (ii) 24 hours after dosing with a skin wash after 6 hours, (iii) 72 hours after dosing with a skin wash after 6 hours and (iv) 120 hours after dosing with a skin wash after 6 hours.

Each rat was lightly anaesthetised using Isoflourane. Any residual dose preparation was collected from each application site by a final washing of the skin with liquid soap solution (nominally a 3% aqueous solution of Dove) and water applied using pieces of pre-wetted natural sponge. Typically 6 sponges of soap solution and 6 with water followed by two dry sponges to remove any residual water were used to wash each application site, including the inner surface of the O-ring. Care was taken with the first few sponges to avoid any contact with the device to prevent the transfer of test substance residues from the skin surface. For each rat, all sponges and washes were collected into a single container.

Following terminal skin washing, each rat was killed by exsanguination under terminal anaesthesia and the blood sample taken into heparinised tubes followed by cervical dislocation. The application site and an annular ring of untreated skin was excised and pinned out on a board. The O-rings were then detached and transferred to the same container as used for the upper O-rings and gauze for each rat. The skin beneath each O-ring, together with a surrounding annular ring of untreated skin, was washed and dried with additional sponges and the sponges stored with the other sponges for each rat. Using successive pieces of adhesive tape the application skin site was tape-stripped to remove the *stratum corneum* until the epidermis was visible (the first and second tape strips from each animal were transferred into two separate containers, further tape strips were performed and placed in a third container). The residual skin was similarly retained in a single container for each rat.

Any urine present in the bladder was collected and added to the corresponding excreted sample. The gastrointestinal tract and contents, the liver and kidneys and representative samples of abdominal fat and muscle were removed from each rat and retained. Each residual carcass was retained for analysis. Each sample was transferred to a pre-labelled container and stored with the residual carcasses at approximately -20°C, with the exception of whole blood samples, which were refrigerated.

Following the removal of rats and the collection of excreta, metabolism cages were washed with an appropriate volume of 1.0M sodium carbonate solution. The internal surface of each metabolism cage was then wiped with cotton wool soaked in 1.0M sodium carbonate solution (these were retained). Finally, each cage was washed with an appropriate volume of ethanol:water (50:50 v/v). These washings were stored at room temperature prior to analysis.

Sample preparation, analysis and measurement of radioactivity: Details of sample preparation are provided in the table below.

Table 2: Sample Preparation Details

Sample type	Preparation method
Diluted solutions of the dose preparation, urine, cage wash, plasma and other solutions, including solvent extracts and tissue digests.	Liquid scintillation counting (LSC).
Faeces	Homogenised to a paste following the addition of a small amount of water. Small samples were analysed by sample oxidation.
Dose pipette tips, O-rings and gauze covers.	Extraction with acetonitrile.
Sponges used to wash application sites, *stratum corneum* on tape strips and skin.	Solubilised in Soluene tissue digestant.
Whole blood	Sample oxidised
Gastrointestinal tract and contents, abdominal fat, kidney, liver, muscle.	Homogenised (by scissor mincing) for sample oxidation.
Residual carcasses	Solubilised in tissue digestant at 40°C.

Table was taken from page 21 of the study report

The radioactivity associated with the dosing formulations, plasma, urine, cage washings, extracts of pipette tips, extracts of O-rings and gauze covers, samples of Soluene and carcass digests were determined directly by liquid scintillation counting of known weights of samples. Samples were mixed with Ultima Gold scintillant (Hionic Fluor was used for carcass digests) and counted using a liquid scintillation counter.

Aliquots of whole blood, abdominal fat, kidney, liver, muscle, gastro-intestinal tract and faecal homogenate were combusted in oxygen using a Packard automated sample oxidiser, and the $^{14}CO_2$ produced was trapped with the carbon dioxide absorbent Carbosorb E^+, which was mixed with the scintillant Permafluor E^+ prior to liquid scintillation counting. Combustion efficiency was in the range 95-105% for all samples oxidised.

Radioactivity in all samples was quantified directly by liquid scintillation counting (LSC) using a Packard liquid scintillation counter with automatic external standard quench correction. After choosing the optimal channel setting, quench correction curves were prepared from radiochemical standards. The validity of the curves was checked throughout the experiments. Radioactivity with less than twice background counts was considered to be below the limit of accurate quantification.

Total radioactivity data were collected and calculated using the validated DEBRA computerised data acquisition system and Excel software.

The limit of detection (LOD) of radioactivity in each sample was taken as equal to twice the scintillation counter background rate, in disintegrations per minute, determined by the use of reagent blanks for each batch of scintillation counts

Radioactivity recovered from the application site skin washings, the O-rings and nonocclusive gauze covers and the first two tape strips of the *stratum corneum* from the application site skin was considered to be unabsorbed. Radioactivity present in the remaining tape strips of the *stratum corneum* and in the underlying application site skin is considered as potentially absorbable as it is recognised that some of this residue may be absorbed beyond the duration of exposure investigated. The absorbed dose included the radioactivity in urine, faeces, cage wash, residual carcass, g.i. tract including contents, abdominal fat, kidney, liver and muscle.

The carcass values reported as percentages of dose include measurements of radioactivity in the blood samples collected at termination.

RESULTS AND DISCUSSION

Signs and Symptoms of Toxicity: Not reported.

Summary Tables: Group mean results showing the distribution of radioactivity after 6, 24, 72 and 120 hours are presented as percentages of the applied dose in the tables below for the formulation concentrate and the 1/100 and 1/400 aqueous dilutions.

Table 3: Distribution of bicyclopyrone Following the Dermal Application of 20 mg [^{14}C]-bicyclopyrone an SL Formulation Concentrate (A16003E) Expressed as Percentage of Dose

	Normalized percentage of applied dose recovered (mean of *n* rats)			
	6 hours (n=3)*	**24 hours (n=4)**	**72 hours (n=4)**	**120 hours (n=4)**
6 hour skin wash	76.15	89.86	93.43	89.81
Terminal skin wash	-	0.33	0.14	0.04
O-rings	0.14	0.23	0.10	0.08
Stratum corneum (1)	0.01	<0.01	<0.01	<0.01
Stratum corneum (2)	0.01	<0.01	<0.01	<0.01
Total unabsorbed	**76.30**	**90.42**	**93.67**	**89.94**
Stratum corneum (3)	0.01	0.01	<0.01	0.01
Application site skin	7.02	1.33	1.47	0.81
Potentially absorbable	**7.03**	**1.34**	**1.47**	**0.81**
Urine	4.72	4.49	2.84	5.71
Faeces	0.22	1.67	1.53	3.02
Cage wash	0.26	0.29	0.10	0.08
Abdominal fat	0.07	0.01	<0.01	<0.01
Kidney	0.07	0.01	0.01	0.01
Liver	0.72	0.14	0.17	0.18
Muscle	0.76	0.04	<0.01	<0.01
GI tract + contents	5.23	0.77	0.03	<0.01
Carcass + blood	4.42	0.85	0.20	0.23
Absorbed dose	**16.46**	**8.24**	**4.86**	**9.23**
Total absorbed dose (sum of absorbed and potentially absorbable)	**23.49**	**9.58**	**6.33**	**10.04**
Total recovered	**100.00**	**100.00**	**100.00**	**100.00**

GI (Gastro-intestinal)

Stratum corneum (1) and (2) = tape strips 1 and 2, respectively.

Stratum corneum (3) = remaining tape strips

* Rat 2 is excluded from the group mean because of an anomalously low skin wash recovery

Table was taken from page 33 of the study report

Table 4: Distribution of bicyclopyrone Following the Dermal Application of 0.20 mg [^{14}C]-bicyclopyrone in a 1/100 dilution of SL Formulation (A16003E) Expressed as Percentage of Dose

	Normalized percentage of applied dose recovered (mean of *n* rats)			
	6 hours (n=4)	**24 hours (n=4)**	**72 hours (n=4)**	**120 hours (n=4)**
6 hour skin wash	78.19	83.38	84.98	81.45
Terminal skin wash	-	2.24	0.84	0.60
O-rings	0.04	0.03	0.11	0.07
Stratum corneum (1)	0.02	0.02	0.01	0.03
Stratum corneum (2)	0.03	0.05	0.01	0.03
Total unabsorbed	**78.28**	**85.72**	**85.94**	**82.17**
Stratum corneum (3)	0.06	0.15	0.07	0.07
Application site skin	14.77	9.14	8.42	10.82
Potentially absorbable	**14.82**	**9.29**	**8.49**	**10.89**
Urine	0.58	0.26	0.40	0.81
Faeces	0.02	0.07	0.15	0.29
Cage wash	0.05	0.06	0.08	0.20
Abdominal fat	0.02	0.02	0.02	0.01
Kidney	0.37	0.42	0.37	0.48
Liver	4.79	3.86	3.84	4.54
Muscle	0.35	0.13	0.24	0.21
GI tract + contents	0.31	0.05	0.08	0.04
Carcass + blood	0.42	0.14	0.40	0.35
Absorbed dose	**6.89**	**5.00**	**5.57**	**6.93**
Total absorbed dose (sum of absorbed and potentially absorbable)	**21.71**	**14.29**	**14.06**	**17.82**
Total recovered	**100.00**	**100.00**	**100.00**	**100.00**

GI (Gastro-intestinal)

Stratum corneum (1) and (2) = tape strips 1 and 2, respectively.

Stratum corneum (3) = remaining tape strips

Table was taken from page 34 of the study report

Table 3: Distribution of bicyclopyrone Following the Dermal Application of 0.05 mg [^{14}C]-bicyclopyrone in a 1/400 dilution of SL Formulation (A16003E) Expressed as Percentage of Dose

	Normalized percentage of applied dose recovered (mean of *n* rats)			
	6 hours (n=4)	**24 hours (n=4)**	**72 hours (n=4)**	**120 hours (n=4)**
6 hour skin wash	75.77	75.63	80.78	79.52
Terminal skin wash	-	2.46	1.24	0.42
O-rings	0.11	1.22	0.08	0.16
Stratum corneum (1)	0.05	0.07	0.03	0.02
Stratum corneum (2)	0.08	0.19	0.01	0.07
Total unabsorbed	**76.00**	**79.56**	**82.12**	**80.19**
Stratum corneum (3)	0.11	0.11	0.06	0.06
Application site skin	21.36	15.25	11.67	9.15
Potentially absorbable	**21.48**	**15.35**	**11.73**	**9.21**
Urine	0.07	0.29	0.38	0.96
Faeces	0.01	0.07	0.10	0.76
Cage wash	<0.01	<0.01	0.06	0.02
Abdominal fat	<0.01	<0.01	0.01	0.02
Kidney	0.56	1.00	0.91	1.04
Liver	1.44	3.48	4.24	7.29
Muscle	0.27	0.12	0.23	0.47
GI tract + contents	0.08	<0.01	0.07	<0.01
Carcass + blood	0.11	0.14	0.18	0.04
Absorbed dose	**2.53**	**5.09**	**6.15**	**10.61**
Total absorbed dose (sum of absorbed and potentially absorbable)	**24.01**	**20.44**	**17.88**	**19.82**
Total recovered	**100.00**	**100.00**	**100.00**	**100.00**

GI (Gastro-intestinal)

Stratum corneum (1) and (2) = tape strips 1 and 2, respectively.

Stratum corneum (3) = remaining tape strips

Table was taken from page 35 of the study report

Table 4: Mean Concentrations of [^{14}C]-bicyclopyrone in Whole Blood and Plasma Following the Dermal Application of an SL Formulation (A16003E)

Collection Time (h)	µg Equivalents of bicyclopyrone/g (mean of 4 rats)					
	Whole Blood			Plasma		
	concentrate	1/100 dilution	1/400 dilution	concentrate	1/100 dilution	1/400 dilution
6	4.757*	0.004	<0.001	6.995*	0.007	<0.001
24	0.137	<0.001	<0.001	0.269	<0.001	<0.001
72	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
120	0.262	<0.001	<0.001	<0.001	<0.001	<0.001

* Rat 2 is excluded from the group mean because of an anomalously low skin wash recovery

Table was taken from page 36 of the study report

Total Absorbed Dose: Recovery of the applied dose was acceptable. The original group mean percentage recoveries of applied radioactivity at each dose level were 97-108% for formulation concentrate, 109-120% for the 1/100 spray strength dilution and 108–122% for the 1/400 spray strength dilution. The tabulated results, however, have been normalized to 100% recoveries.

The pattern of absorption observed in this study appeared to be different between the formulation concentrate and its two aqueous dilutions. Absorption from the concentrate occurred at a faster rate than from both dilutions, because skin residues were very low by 24 hours and thereafter and absorption did not increase markedly over the 120 hour time course. Despite the exclusion of results for rat 2, the group mean absorption data for the three remaining rats in the 6 hour group remain anomalously high when compared with the other 3 groups of similarly treated rats. Hence, since consistently lower absorption results were observed in the 24, 72 and 120 hour groups, the absorption of 23.5% of the dose after 6 hours is considered to represent an overestimate and the value is taken to be unreliable. Despite some inter-animal variability, absorption between 24 and 120 hours increased only slightly as the low but potentially absorbable skin residues declined.

The rate of absorption of bicyclopyrone from both aqueous dilutions was more pronounced than from the formulation concentrate because the skin appeared to be acting as a reservoir for some of the applied dose of bicyclopyrone for potential subsequent absorption. The capacity of this reservoir was limited for the concentrate retaining only *circa* 1% of the formulation concentrate dose. However, in contrast, higher proportions of the lower aqueous dilution doses were retained.

The extent of radioactivity absorbed in the tissues and excreta from the 1/100 dilution amounted to 6.9% of the dose after 6 and 120 hours. Over the same time course residues in the skin (potentially absorbable) declined from 14.8% to 10.9%, resulting in overall absorption values (calculated as the sum of absorbed and potentially absorbed) ranging from 14-22%.

In the 1/400 aqueous dilution, absorption increased steadily from 2.5% immediately after the 6 hour exposure interval, to 5.1% after 24 hours, 6.2% after 72 hours and 10.6% after 120 hours (24.01, 20.44, 17.88 and 19.82% with potentially absorbable). Over the same time course the potentially absorbable skin residue declined steadily from 21.5% to 9.2% resulting in overall absorption values (calculated as the sum of absorbed and potentially absorbed) ranging from 18-24%.

INVESTIGATOR'S CONCLUSIONS: Following a 6 hour dermal exposure to bicyclopyrone SL formulation concentrate (A16003E) and to 1/100 and 1/400 spray strength dilutions thereof, most of the applied dose was readily removed from the skin surface by mild skin washing. In all dose groups residues remained in the skin after washing and thereafter declined in concentration over the 120 hour assessment period, **with a corresponding increase in excreta and tissues, indicating continued absorption of the chemical after ceasing exposure**.

Total absorption of bicyclopyrone (calculated as the sum of absorbed and potentially absorbed doses) over a time course of 120 hours accounted for 10.0%, 17.8%, and 19.8% of the concentrate, 1/100 dilution, and 1/400 dilution, respectively.

REVIEWER COMMENTS:

In the results section, the absorbed and the potentially absorbed doses are considered together in accordance with current EPA policy. The dermal absorption values originally calculated in the study report did not include radioactivity in the stratum corneum (minus the first 2 tape strips) and it is the opinion of the agency that the application site skin and that these should be included since they are considered bioavailable and may potentially be absorbed. The final dermal absorption values were thus calculated combining the absorbed + potentially absorbed for each dose level and time point.

This study is classified as totally reliable **(acceptable/guideline)** as a combined *in vivo* dermal absorption study in rats (OPPTS 870.7600). EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

(Read H, 2009)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Anwar Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/17/15
EPA Reviewer: Monique Perron, S.D. **Signature:** Monique Perron
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/17/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: 28-Day Dietary Immunotoxicity Study in Mice (OPPTS 870.7800 (1998)

TEST MATERIAL (PURITY): NOA449280 (purity 94.5%)

SYNONYMS: Bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Eapen A, 2012. NOA449280: A 28 Day dietary immunotoxicity study in CD-1 female mice. WIL Research Laboratories, LLC, 1407 George Road, Ashland, OH 44805-8946, USA and ImmunoTox®, Inc., Virginia BioTechnology Research Park, 800 East Leigh Street, Suite 209, Richmond, VA 23219, USA. Laboratory Report No. WIL-639059. 19 April 2012. Unpublished. Syngenta File No. NOA449280/11151.MRID 47842008

SPONSOR: Syngenta Crop Protection, LLC, 410 Swing Road, Post Office Box 18300, Greensboro, NC 27419-8300 USA

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

EXECUTIVE SUMMARY

Bicyclopyrone (NOA449280, purity 94.5%), was administered *ad libitum* in the diet for a minimum of 28 consecutive days to 3 groups of approximately 7 week old, Crl:CD1(ICR) female mice (groups 2-4) at dietary concentrations of 50, 500 and 5000 ppm, respectively (actual doses were 10.6, 107.2 and 1192 mg/kg/day [F]). A vehicle control group (group 1) was given untreated diet. A positive control group was dosed with cyclophosphamide (CPS), via intraperitoneal injection (50 mg/kg/day, dose volume 10 mL/kg/day) for 4 consecutive days. The vehicle and positive control groups (Groups 1 and 5, respectively) were offered the basal diet on a comparable regimen as the bicyclopyrone treated groups. Additionally, all mice were immunised with an intravenous injection of sheep red blood cells (sRBC) on study day 24, approximately 96 hours prior to the scheduled necropsy. Each group consisted of 10 females.

All animals were observed twice daily for mortality and moribundity. Clinical examinations, body weights and food consumption were recorded throughout the study. All animals were killed on day 28.

Complete necropsies were conducted on all animals. A bone marrow smear (right femur) and the liver, mesenteric lymph nodes, Peyer's patches (GALT), spleen, and thymus were collected from all animals. The liver, spleen, and thymus were weighed. Spleens were placed in EBSS/HEPES buffer and spleen cell suspensions prepared. Spleen cell counts were performed and the number of specific IgM antibody forming cells directed towards the sRBC were determined.

All animals survived to the scheduled necropsy. There were no treatment-related effects on body weights, food consumption, clinical observations, or macroscopic findings. There were no test substance-related effects on absolute, adjusted, or relative spleen or thymus weights, or spleen cell numbers. There were no effects attributed to bicyclopyrone on the specific activity or total activity of splenic IgM antibody-forming cells to the T cell-dependent antigen sRBC.

Statistically significantly higher test substance-related higher liver weights were noted in the 5000 group (↑23%) as compared to the vehicle control group. These increases were consistent with the results of previous studies and were attributed to bicyclopyrone exposure; however, they are not considered adverse.

For systemic toxicity, the NOAEL is 5000 ppm (1192 mg/kg/day). The systemic LOAEL was not established.

Lower mean absolute and adjusted spleen and thymus weights were noted for the CPS (positive control) group when compared to the vehicle control group (↓42 and ↓39%). Additionally, CPS administration produced statistically significantly lower spleen cell numbers (56% lower), specific activity (100% lower), and total spleen activity (100% lower) of IgM antibody-forming cells when compared to the vehicle control group. These effects are consistent with the known immunosuppressant effects of CPS and validate the sensitivity of the assay.

For immunotoxicity, the NOAEL is 5000 ppm (1192 mg/kg/day). The LOAEL for immunotoxicity was not established.

This 28-day dietary immunotoxicity study in the mouse is totally reliable (**acceptable/guideline**) and satisfies guideline requirement for an immunotoxicity study (OPPTS 870.7800).

COMPLIANCE: Signed and dated No Data Confidentiality, GLP, and Quality Assurance statements were provided.

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone (NOA449280)
Description:	Technical, light brown powder
Lot/Batch number:	SEZ3AP006/Milled
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Reanalysis date March 2011

Structure:

Vehicle and/or positive control: Control group: plain diet.
Positive control: cyclophosphamide monohydrate (CPS), in phosphate-buffered saline (PBS) via intraperitoneal injection (50 mg/kg/day, dose volume 10 mL/kg/day).

Test Animals:

Species	Mouse, female
Strain	Crl:CD-1(ICR)
Age/weight at dosing	Approximately 7 weeks/21.5-27.3 g
Source	Charles River Laboratories, Inc., Raleigh, NC, USA
Housing	Individually in suspended stainless steel, wire-mesh cages
Acclimatisation period	14 days
Diet	PMI LLC Certified Rodent LabDiet 5002 (meal) *ad libitum*
Water	Reverse osmosis treated municipal water *ad libitum*
Environmental conditions	Temperature: 21.2-22.1°C Humidity: 41.0-46.9% Air changes: At least 10 changes per hr Photoperiod: 12 hrs dark / 12 hrs light:

Study Design and Methods:

In-life dates: Start: 22 December 2009 19 January 2010

Animal assignment: Animals were assigned to the study by a computerised randomisation procedure. Individual body weights at randomisation were within ±20% of the mean.

Table 1: Study design

Test group	Dietary concentration of bicyclopyrone (ppm)	Number of female mice
1	0 (control)	10
2	50	10
3	500	10
4	5000	10
5	0 (CPS positive control)	10

Table was taken from page 21 of the study report

Dose selection rationale: The dose levels were based on the results of a previous 13 week dietary bicyclopyrone toxicity study using CD-1 mice (CRL Report 28445). In this study, the high dose (5000 ppm), which exceeded the previously determined lowest-observed-adverse-effect level (LOAEL) of 3500 ppm, was anticipated to approximate the limit dose of 1000 mg/kg/day. The mid dose (500 ppm; approximately 120 mg/kg/day) was slightly higher than the previously established no-observed-adverse-effect level (NOAEL) of 100 ppm, and the low dose (50 ppm; approximately 12 mg/kg/day) was slightly lower than the previously established NOAEL.

Diet preparation: Diets were prepared approximately weekly. Appropriate amounts of bicyclopyrone was added to a portion of the PMI Nutrition International, LLC Certified Rodent LabDiet® 5002 (meal) for a specific dose group, blended in a mixer for each test substance concentration, and the resulting premix was mixed thoroughly with the remaining feed to obtain the appropriate dietary concentration. Diets were stored at room temperature.

Stability analysis: The analysed dietary formulations were found to contain 92.9%-103% of the test substance after one week. The test substance was not detected in the basal diet that was administered to the vehicle control group.

Homogeneity analysis: Overall mean homogeneity values (% of target ± RSD) for the 50 ppm preparations were 1.5% and 1.4% for Weeks 1 and 3, respectively. Overall mean homogeneity values for the 500 ppm preparations were 1.1% and 2.4% for Weeks 1 and 3, respectively. Overall mean homogeneity values for the 5000 ppm preparations were 0.63% and 1.2% for Weeks 1 and 3, respectively.

Concentration analysis: All concentration verification results were acceptable. Mean concentrations from Weeks 1 and 3 were 93% and 51% of the target concentration for the 50 ppm formulations, respectively; 93.6% and 103% of the target concentration for the 500 ppm formulations, respectively, and 92.9% and 98.7% of the target concentration for the 5000 ppm formulations, respectively.

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable, provided that the cited stability study did indicate that the test compound was stable under conditions of the study.

Experimental procedures: Bicyclopyone (NOA449280, purity 94.5%), was administered *ad libitum* in the diet for a minimum of 28 consecutive days to 3 groups of Crl:CD1(ICR) female mice (groups 2-4) at dietary concentrations of 50, 500 and 5000 ppm, respectively. A vehicle control group (group 1) was given untreated diet. A positive control group was dosed with cyclophosphamide (CPS), via intraperitoneal injection (50 mg/kg/day, dose volume 10 mL/kg/day) for 4 consecutive days. The vehicle and positive control groups (Groups 1 and

5, respectively) were offered the basal diet on a comparable regimen as the bicyclopyrone treated groups. Additionally, all mice were immunised with an intravenous injection of 0.2 mL of 1 x 10^8 sheep red blood cells (sRBC) (buffered with EBSS and HEPES) on study day 24, approximately 96 hours prior to the scheduled necropsy. Each group consisted of 10 females.

Observations: Clinical examinations were performed once daily for all animals. Detailed physical examinations were conducted on all animals approximately weekly, beginning at least 1 week prior to randomisation, at the time of randomisation, and on the day of the scheduled necropsy.

Body weight: Individual body weights were recorded twice weekly throughout the study beginning at least 1 week prior to randomisation, including at the time of animal selection for randomisation, on study day 0, and on the day of the scheduled necropsy.

Food consumption and test substance intake: Individual food consumption was recorded approximately weekly during the pretest period (beginning at least 1 week prior to randomisation) and throughout the study. Food intake was calculated as g/animal/day for each interval.

The mean amounts of bicyclopyrone consumed (mg/kg/day) by each treatment group were calculated from the mean food consumed (g/kg of body weight/day using the average of the first and last body weight during the week) and the appropriate target concentration of bicyclopyrone in the food (mg/kg).

Investigations *post mortem:*
Clinical pathology: Blood samples were collected following euthanasia by carbon dioxide inhalation from the inferior vena cava of all animals on the day of the scheduled necropsy. The blood samples (approximately 0.1 mL) were collected into individual tubes containing potassium EDTA as an anticoagulant for the preparation of blood smears for possible future evaluation

IgM antibody analysis: Blood samples were collected following euthanasia by carbon dioxide inhalation from the inferior vena cava of all animals at the scheduled necropsy. The blood samples (approximately 0.75 mL, if possible) were collected into individual borosilicate glass tubes and processed to obtain serum samples. The serum was transferred to cryovials and stored frozen at approximately -70ºC for possible future IgM antibody analysis.

Macroscopic examination: A complete necropsy was conducted on all animals. Mice were killed by carbon dioxide inhalation followed by blood sample collection. The necropsies included, but were not limited to, examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, including viscera.

The following tissues and organs were collected from animals at the scheduled necropsy for possible future histopathological examination: bone marrow smears, liver, lymph node (mesenteric), Peyer's patches, spleen and thymus.

Organ weights: The following organs were weighed from all animals at the scheduled necropsy: liver, spleen (wet weight), and thymus.

Spleen processing for immunotoxicological evaluation: Spleens were collected from all animals at the scheduled necropsy immediately following blood collection. Individual spleens were placed into individual, tared tubes maintained on ice containing Earle's Balanced Salt Solution (EBSS) with 15 mM HEPES and supplemented with gentamicin as a bacteriostat. Each tube was then weighed to provide a 'wet' weight for each spleen. Spleen samples from group 1-4 animals were randomised and coded for analysis. This was done to ensure that the analyst was unaware from which treatment group the spleen sample had been collected. Spleen samples from group 5 were labelled as positive control samples for analysis. The spleen samples were placed on crushed ice and shipped to ImmunoTox®, Inc. for the antibody plaque-forming cell (PFC) assay.

The spleen samples were processed into single-cell suspensions. The cell suspensions were centrifuged and resuspended in EBSS with HEPES. Spleen cell counts were performed using a Model Z1 Coulter Counter®. Viability of splenocytes was determined using propidium iodide and the Coulter® EPICS® XL-MCL Flow Cytometer. The PFC assay served to determine the number of specific IgM antibody forming cells directed towards sRBC and was a modification of the Jerne plaque assay (***Jerne et al., 1963***).

Positive control: The positive control substance (CPS) was administered to group 5 via intraperitoneal injection once daily on study days 24 through 27. The positive control substance dose level was 50 mg/kg/day and the dose volume was 10 mL/kg/day. On study day 24, approximately 96 hours prior to the scheduled necropsy, all animals were immunised via a lateral tail vein by intravenous injection of 0.2 mL of 1 x 10^8 sRBC in EBSS with HEPES. Group 5 animals received the sRBC immunisation prior to the positive control dose administration on study day 24.

Statistics: All statistical tests were performed using appropriate computing devices or programs. Analyses were generally conducted using two-tailed tests for minimum significance levels of 1% and 5%, comparing each NOA449280-treated group to the vehicle control group. For the purpose of data interpretation, statistical significance was not considered automatically to imply immunotoxicological significance. Conversely, the absence of a statistically significant comparison was not considered solely to imply the lack of a biologically relevant effect. The immunotoxicological relevance of any treatment-related findings was based, in part, on a weight of evidence analysis of the data.

RESULTS AND DISCUSSION

Mortality: None of the animals died.

Clinical observations: There were no clinical abnormalities recorded during the study

Body weight and weight gain: There were no treatment-related differences from control for body weight or body weight gain during the study. See table 2.

Table 2: Intergroup comparison of bodyweights

	Dietary concentration of bicyclopyrone (ppm)				
	Females				
Day	0	50	500	5000	CPS (50 mg/kg)
0	24.1 ± 1.57	24.4 ± 1.14	24.0 ± 1.11	24.0 ± 1.42	24.0 ± 1.15
7	24.9 ± 1.67	25.2 ± 1.52	25.6 ± 1.46	24.7 ± 1.77	25.1 ± 1.33
14	25.5 ± 1.69	26.5 ± 2.00	26.7 ± 1.86	25.4 ± 2.29	26.1 ± 1.90
21	26.1 ± 1.88	26.9 ± 1.91	27.0 ± 1.78	25.7 ± 2.11	26.1 ± 1.98
28	27.7 ± 1.89	28.0 ± 1.82	28.3 ± 1.73	27.4 ± 2.08	26.5 ± 2.06

Data were taken from pages 36 to 39 of the study report.

Food consumption and compound intake: Food consumption was unaffected by bicyclopyrone administration.

Table 3: Average test substance consumption (mg/kg/day)

Target dietary concentration (ppm)	Average bicyclopyrone consumption (mg/kg/day)
50	10.6
500	107.2
5000	1192.3

Data were taken from page 27 of the study report.

Macroscopic examination: There were no bicyclopyrone related macroscopic findings at the scheduled necropsy.

Organ weights: Test substance-related higher liver weights were noted in all dose groups as compared to the vehicle control group (significantly higher mean absolute and adjusted liver weights at 5000 ppm, and significantly higher mean adjusted liver weights at 50 and 500 ppm). These increases were consistent with the results of previous studies and were attributed to bicyclopyrone exposure; however, they are considered not to be adverse or immunotoxicologically relevant. See table 4.

Table 4: Absolute and Adjusted Liver Weights (g)

Dose Level of bicyclopyrone (mg/kg/day)					
Females					
	0	50	500	5000	CPS (50 mg/kg)
Absolute	1.33 ± 0.15	1.52 ± 0.23	1.57 ± 0.27	1.64* ± 0.25 (↑23%)	1.38 ± 0.21
Adjusted	1.34	1.51* (↑13%)	1.53* (↑14%)	1.68** (↑25%)	1.42* (↑6%)
Relative (g/100g bw)	4.8 ± 0.34	5.4 ± 0.53 (↑13%)	5.5 ± 0.76 (↑16%)	5.9 ± 0.59 (↑24%)	5.2 ± 0.56 (↑8%)

Data were taken from page 48

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Antibody plaque-forming cell (PFC) assay: A summary of the results from the PFC assay are presented in table 5. There was no significant effect on spleen cell numbers for the animals treated with bicyclopyrone.

Bicyclopyrone administration did not suppress the humoral immune response when evaluated as either Specific Activity (PFC/10^6 Spleen Cells) or Total Spleen Activity (PFC/Spleen).

As expected, CPS administration produced a statistically significant decrease in spleen cell numbers (56% lower), Specific Activity (100% lower), and Total Spleen Activity (100% lower) when compared to the vehicle control group.

Table 5: Summary of body weight, spleen weight, thymus weight, and PFC assay results for the CD-1 female mice exposed to bicyclopyrone

	Dietary concentration of bicyclopyrone (ppm)				
Parameter	**0 (Vehicle Control)**	**50**	**500**	**5000**	**CPS (50 mg/kg)**
Terminal Body Weight	-	NS	NS	NS	NS
Spleen Weights-(Absolute)	-	NS	NS	NS	-42%
Spleen Weights-(Relative to Body Weight)	-	+28%	NS	NS	-39%
Thymus Weights (Absolute)	-	NS	NS	NS	-58%
Thymus Weights (Relative to Body Weight)	-	NS	NS	NS	-53%
Spleen PFC Response:					
Spleen Cell Number	-	NS	NS	NS	-56%
IgM PFC/10^6 Spleen Cells	-	NS	NS	NS	-100%
IgM PFC/Spleen ($x10^3$)	-	NS	NS	NS	-100%

Table was taken from page 97 of the study report
NS= No significant exposure-related effect, as compared to vehicle controls
Vehicle Control= Basal diet (untreated with test substance NOA449280)
CPS=Cyclophosphamide (positive control), which was administered on days 24-27
Spleen Antibody (IgM) forming cell (PFC) responses=expressed as Spleen Cell Number, Specific Activity (PFC/10^6 spleen) and Total Spleen Activity (PFC/spleen)

Natural killer cell activity assay: There were no indications in this study that bicyclopyrone is immunosuppressive. Similarly, data from subchronic, chronic, and reproductive toxicity studies of bicyclopyrone in the rat, mouse and dog (as appropriate) do not provide any

evidence of an effect of bicyclopyrone on the immune system [refer to Minutes and Memorandum of Understanding of the Meeting (November 3, 2009) with the Dose Adequacy and Review Team (DART) and Syngenta Crop Protection: Review of the Proposed Immunotoxicity Testing Parameters for NOA449280 (Bicyclopyrone), dated 7 December 2009]. From these findings Syngenta concludes that there is no evidence of immunosuppression associated with bicyclopyrone, and therefore the need to conduct additional immunotoxicity assessments, specifically a natural killer (NKC) cell activity assay, is not identified at this time.

Positive control: Data for CPS on specific immune system function are presented in table 5. Lower mean absolute and adjusted spleen and thymus weights were noted for the CPS (positive control) group when compared to the vehicle control group. Additionally, CPS administration produced statistically significantly lower spleen cell numbers (56% lower), specific activity (100% lower), and total spleen activity (100% lower) of IgM antibody-forming cells when compared to the vehicle control group. These effects are consistent with the known immunosuppressant effects of CPS and validate the sensitivity of the assay.

CONCLUSION: Treatment with bicyclopyrone in the diet for a minimum of 28 days did not result in suppression of the humoral component of the immune system. The no-observed-effect level (NOEL) for immune suppression was 5000 ppm (equivalent to 1192.3 mg/kg/day). The only effect attributed to bicyclopyrone was higher liver weights noted for the 50, 500, and 5000 ppm groups; this effect was considered not to be adverse. There were no indications in this study that bicyclopyrone was immunotoxic.

Similarly, data from subchronic, chronic, and reproductive toxicity studies of bicyclopyrone in the rat, mouse and dog, as appropriate, did not provide any evidence of an effect of bicyclopyrone on the immune system, as indicated in the Minutes and Memorandum of Understanding from the 03 November 2009 Meeting with the US EPA Dose Adequacy and Review Team (DART) and Syngenta (dated 07 December 2009).

Based on data from all relevant studies, Syngenta concludes that there is no evidence of immunotoxicity associated with bicyclopyrone, and therefore, no need to conduct additional immunotoxicity assessments, specifically a natural killer cell (NKC) activity assay.

REVIEWER COMMENTS:

For systemic toxicity, the NOAEL is 5000 ppm (1192 mg/kg/day). The systemic LOAEL was not established.

For immunotoxicity, the NOAEL is 5000 ppm (1192 mg/kg/day). The LOAEL for immunotoxicity was not established.

APVMA/OCS (Australia) proposed inclusion of the quantitative data (see pages 113 and 114 of study report). Based upon the lack of toxicity in this study, however EPA does not the value in adding this data to this data evaluation record.

This 28-day dietary immunotoxicity study in the mouse is totally reliable (**acceptable/guideline**) and satisfies guideline requirement for an immunotoxicity study (OPPTS 870.7800). EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

Reference:

Jerne, N. K.; Nordin, A. A.; Henry, C. The Agar Plaque Technique for Recognizing Antibody-Producing Cells. In: *Cell Bound Antibodies*. Amos, B., Koprowski, H., Eds.; Wistar Institute Press: Philadelphia, PA **1963**.

(Eapen A, 2012)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:** Ann Y. Dunbar
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/18/15
EPA Reviewer: Greg Akerman, Ph.D. **Signature:**
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/18/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Effect on Rat Thyroid Peroxidase Activity *in vitro:* No applicable guidelines

TEST MATERIAL (PURITY): Bicyclopyrone (purity 94.5%)

SYNONYMS: NOA449280; bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Lake B (2012). Bicyclopyrone: Effect on rat thyroid peroxidase activity *in vitro*. Leatherhead Food Research (LFR), Molecular Sciences Department, Randalls Road, Leatherhead, Surrey, KT22 7RY, United Kingdom. Laboratory Report No. 5492/1/1/2012, 06 February 2012. Unpublished. Syngenta File No. NOA449280/11141.MRID 47841989

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom.

COMPLIANCE: This study was not conducted according to Good Laboratory Practice Standards as defined by OECD. No claim of GLP compliance was made for this study.

EXECUTIVE SUMMARY

The aim of this study was to evaluate the effect of bicyclopyrone (0 [control], 0.1, 10 and 100 µM) on rat thyroid peroxidase activity *in vitro*. A pooled thyroid gland microsomal preparation from five rats was assayed for thyroid peroxidase activity by determining the monoiodination of L-tyrosine. As a positive control, the effect of 6-propyl-2-thiouracil (PTU; 1 and 10 µM) on rat thyroid peroxidase activity was also determined.

Treatment with bicyclopyrone had no significant effect on rat thyroid peroxidase activity at any concentration tested. Treatment with 10 µM 6-propyl-2-thiouracil (PTU) resulted in a 100% inhibition of thyroid peroxidase activity, whereas treatment with 1 µM had no significant effect.

Bicyclopyrone was negative for inhibition of rat thyroid peroxidase *in vitro*.

This study is classified as totally reliable **(acceptable/non-guideline)**.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone
Description:	Technical, brown beige powder
Lot/Batch number:	SEZ3AP006/Milled
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Reanalysis date end of September 2013

Structure:

Vehicle and/or positive control: Dimethyl sulphoxide (DMSO) / 10 µM 6-propyl-2-thiouracil (PTU).

Test Animals:

Species	Rat
Strain	Wistar Han
Weight	271-286 g
Source	Charles River UK Ltd., Margate, Kent, CT9 4LT, UK.
Housing	Not reported
Acclimatisation period	At least 5 days
Diet	*ad libitum*
Water	*ad libitum*
Environmental conditions	Not reported

Study Design and Methods:

In-life dates: Start: 04 October 2011 14 November 2011

Preparation of thyroid gland microsomes: Five male Wistar Han rats were killed by carbon dioxide asphyxiation and the thyroid gland attached to the trachea was immediately removed and snap frozen in dry ice and stored at -70°C or below until required. The trachea/thyroid glands were thawed and each thyroid gland dissected from the attached trachea. A whole homogenate of the pooled thyroid glands from the 5 rats was prepared in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4. The thyroid gland whole homogenate was centrifuged at 9000 g average for 20 minutes to obtain the postmitochondrial supernatant which was subsequently centrifuged at 105,000 g average for 60 minutes to separate the microsomal fraction from the cytosol. The pooled thyroid gland microsomal fraction was resuspended in fresh homogenising medium. Aliquots of the pooled thyroid gland microsomal fraction were stored at -70°C or below and were thawed once only for the determination of thyroid peroxidase activity.

Assay of protein content: Thyroid gland microsomal protein content was determined by the general procedure of ***Lowry et al. (1951)***, employing bovine serum albumin as standard. The microsomal protein content of the pooled thyroid gland preparation was calculated to be 17.0 mg protein/g tissue.

Assay of thyroid peroxidase activity: Thyroid peroxidase activity was assayed by determining the monoiodination of L-tyrosine. Incubations contained 150 µM L-tyrosine, 150 µM potassium iodide, 30 µg thyroid microsomal protein, either bicyclopyrone or PTU dissolved in dimethyl sulphoxide (2.5 µL/incubation) and 0.1 M phosphate buffer pH 7.4 in a total volume of 0.25 mL. The bicyclopyrone concentrations studied were 0 (control), 0.1, 10 and 100 µM, whereas the PTU concentrations studied were 1 and 10 µM. The concentrations of bicyclopyrone selected were based on approximate *in vivo* concentrations following dietary administration to rats for up to 2 years. After a 10 minute preincubation in a shaking water bath at 37°C, the reaction was initiated by the addition of 200 µM hydrogen peroxide. Blank incubations (to correct for non enzymatic formation of 3-iodo-L-tyrosine) contained all additions except for thyroid gland microsomes. After a 10 minute incubation in a shaking water bath at 37°C the reaction was terminated and levels of 3-iodo-L-tyrosine in deproteinised supernatants determined by ultra performance liquid chromatography-mass spectrometry-mass spectrometry (UPLC-MS-MS). Under these conditions the rate of formation of 3-iodo-L-tyrosine was linear with respect to both incubation time and protein concentration and the formation of 3,5-diiodo-L-tyrosine in control incubations was <5% of the formation of 3-iodo-L-tyrosine.

Statistics: Data were summarised in the form of mean and standard deviations (SDs) of the mean. Enzyme activity data were tested for normality using the Kolmogorov-Smirnov test (level of significance determined to be at $p<0.10$) and heterogeneity using Bartlett's test (level of significance $p<0.01$). Where transformations were required all were successful. Control and bicyclopyrone treated groups and control and PTU treated groups were subjected to a one-way analysis of variance. Comparisons between control and bicyclopyrone treated groups were made using two-sided Dunnett's tests and between control and PTU treated groups were made using a t-test. In all Dunnett's test and t-test comparisons a probability level of $p<0.05$ was taken to indicate statistical significance.

RESULTS AND DISCUSSION

Results are presented in table 1. Treatment with bicyclopyrone had no significant effect on rat thyroid peroxidase activity at any concentration tested. Treatment with 10 µM 6-propyl-2-thiouracil (PTU) resulted in a 100% inhibition of thyroid peroxidase activity, whereas treatment with 1 µM had no significant effect.

Table 1: Effect of bicyclopyrone and PTU on rat thyroid peroxidise activity

Addition (a)	Thyroid peroxidise activity (nmol/min/mg protein) (b)	Percentage of control values
Control (DMSO only)	3.87 ± 0.267	-
Bicyclopyrone 0.1 µM	4.01±0.102	104
Bicyclopyrone 10 µM	3.95±0.075	102
Bicyclopyrone 100 µM	3.58±0.391	93
PTU 1 µM	3.58±0.161	93
PTU 10 µM	0.00±0.000***	0

(a) bicyclopyrone and PTU were added in DMSO (2.5 µL/incubation).

(b) results are presented as mean±SD for triplicate incubations

*** significantly different from control $p<0.01$

Table was taken from page 12 of the study report

INVESTIGATOR'S CONCLUSION

Bicyclopyrone is not an inhibitor of rat thyroid peroxidase activity *in vitro.*

REVIEWER COMMENTS

The aim of this study was to evaluate the effect of bicyclopyrone (0 [control], 0.1, 10 and 100 µM) on rat thyroid peroxidase activity *in vitro*. A pooled thyroid gland microsomal preparation from five rats was assayed for thyroid peroxidase activity by determining the monoiodination of L-tyrosine. As a positive control, the effect of 6-propyl-2-thiouracil (PTU; 1 and 10 µM) on rat thyroid peroxidase activity was also determined.

Treatment with bicyclopyrone had no significant effect on rat thyroid peroxidase activity at any concentration tested. Treatment with 10 µM 6-propyl-2-thiouracil (PTU) resulted in a 100% inhibition of thyroid peroxidase activity, whereas treatment with 1 µM had no significant effect.

Bicyclopyrone was negative for inhibition of rat thyroid peroxidase *in vitro*.

This study is classified as totally reliable **(acceptable/non-guideline)**. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

REFERENCE: Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

(Lake B 2012)

EPA Reviewer: Anwar Dunbar, Ph.D. **Signature:**
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 03/18/15
EPA Reviewer: Greg Akerman, Ph.D. **Signature:**
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** 3/18/15

TXR#: 0057111

DATA EVALUATION RECORD

PC CODE: 018986 **DP BARCODE:** D425155

STUDY TYPE: Thyroid Mode of Action Study in Rats

TEST MATERIAL (PURITY): Bicyclopyrone (purity 94.5%)

SYNONYMS: NOA449280; bicyclo[3.2.1]oct-3-en-2-one,4-hydroxy-3-[{2-[(2-methoxyethoxy)methyl]-6-(trifluoromethyl)-3-pyridinyl]carbonyl]; 4-hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl)-bicyclo[3.2.1]oct-3-en-2-one; Bicyclopyrone; SYN449280.

CITATION: Donald L (2012). Bicyclopyrone: 28 day dietary thyroid mode of action study in rats. Charles River, Tranent, Edinburgh, EH33 2NE, UK. Laboratory Report No. 32992. 24 August 2012. Unpublished. (Syngenta File No. NOA449280/11266.) MRID 47841990

SPONSOR: Syngenta Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom.

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided.

EXECUTIVE SUMMARY

In an in vivo Mode of Action study (MRID #47841990), the effect of bicyclopyrone (also known as NOA449280) on a number of parameters related to the liver and thyroid function was determined to elucidate the mode of action for thyroid hypertrophy/hyperplasia observed in a combined chronic toxicity and carcinogenicity study in rats. Groups of 75 male Han Wistar Crl:WI (Han) rats were fed diets containing 0, 5, 500 or 5000 ppm (0, 0.5, 41.5 and 399.5 mg/kg/day) bicyclopyrone (purity 94.5%) for a period of up to 28 days. A further group of 30 male Han Wistar rats were fed diets containing 1200 ppm phenobarbital (PB) for up to 8 days, which acted as a positive control with respect to thyroid function testing.

The following were assessed at pre-determined intervals from pretrial until the end of the in-life period: viability, clinical observations, body weights and food consumption. Blood samples were collected from all animals at termination for thyroid function testing, and with the exception of the 1200 ppm PB treated animals (Group 5), tyrosine analysis.

Animals were killed at pre-determined intervals throughout the treatment period and were subjected to a detailed necropsy examination after the completion of treatment. The thyroid gland and liver were collected and weighed from all animals. These organs were processed to slides for histopathological examination. With the exception of the 1200 ppm PB treated

animals (Group 5), the biochemistry of the liver (microsomal protein content and UDPGT activity) was also analysed.

Treatment of male Han Wistar rats with 5, 500 or 5000 ppm bicyclopyrone resulted in dose and time-dependent increases in tyrosine, decreases in tetraiodothyronine (thyroxine; T_4), increases in liver weight and at 5000 ppm, increased incidences of hepatocellular centrilobular hypertrophy. In addition, treatment with 500 or 5000 ppm bicyclopyrone resulted in dose and/or time-dependent increases in hepatic UDGPT activity, decreases in triiodothyronine (T_3) and increased incidences of thyroid follicular cell hypertrophy. Mean body weight gain was lower throughout the treatment period for animals receiving 5000 ppm bicyclopyrone and lower mean food consumption was observed throughout the treatment period for animals receiving 500 or 5000 ppm bicyclopyrone.

Treatment of male Han Wistar rats with 1200 ppm PB resulted in time-dependent decreases in T_4, increases in TSH, increases in liver and thyroid weights, increased incidences of both hepatocellular centrilobular hypertrophy and thyroid follicular cell hypertrophy. This is consistent with previous findings with 1200 ppm PB (Charles River Study No. 459774), demonstrating its suitability as a positive control with respect to thyroid function testing.

This study has demonstrated that dietary treatment of male Han Wistar rats with bicyclopyrone results in increased tyrosine, decreased T_3 and T_4, increased thyroid follicular cell hypertrophy and increased liver weight associated with increased hepatocellular centrilobular hypertrophy and increased hepatic UDPGT activity. These data can be used as part of a weight-of-the-evidence approach to explain the mode of action for the increased incidence of thyroid follicular cell hyperplasia observed in male Han Wistar rats in a 2 year carcinogenicity study.

This study is classified as totally reliable (**acceptable/non-guideline**).

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality

MATERIALS AND METHODS

Materials:

Test Material:	Bicyclopyrone
Description:	Technical, brown beige powder
Lot/Batch number:	SEZ3AP006/Milled
Purity:	94.5% a.i
CAS#:	352010-68-5
Stability of test compound:	Reanalysis date 30 September 2013

Structure:

Vehicle and/or positive control: The test substance was administered via Rat and Mouse (modified) No.1 Diet SQC Expanded Ground. The vehicle control received the diet only. The positive control substance was Phenobarbital Sodium Salt at 1200 ppm in the diet.

Test Animals:

Species	Rat (male)
Strain	Han Wistar Crl:WI (Han)
Age/weight at dosing	Approximately 8-9 weeks/193-315 g
Source	Charles River UK Ltd., Margate, Kent, UK.
Housing	Animals were housed 3 per cage by sex in polycarbonate cages (61 x 43.5 x 24 cm) with stainless steel grid tops, solid bottoms and an integral food hopper.
Acclimatisation period	14 days
Diet	Rat and Mouse (modified) No.1 Diet SQC Expanded Ground *ad libitum*
Water	Public water supply *ad libitum*
Environmental conditions	Temperature: 19-23°C Humidity: 48-59% Air changes: Approximately 10 changes/hour Photoperiod: 12 hrs light/12 hours dark

Study Design and Methods:
Experimental dates: Start: 25 October 2011 09 February 2012

Aim: The purpose of the study was to determine the effect of bicyclopyrone on a number of parameters related to the liver and thyroid function in order to elucidate the mode of action for thyroid hypertrophy/hyperplasia observed in a combined chronic toxicity and carcinogenicity study in rats.

Animal assignment: Cages were racked by treatment group and vertically throughout the rack. All groups were housed on separate racks.

Route and duration of administration: Bicyclopyrone was administered in the diet of male rats (75/dose) for up to 28 days at 0, 5, 500 or 5000 ppm. Animals (15/sacrifice point) were euthanized after 2, 4, 8, 15 or 29 days after the start of treatment. Thirty male rats were administered the positive control in the diet at 1200 ppm for up to 8 days with groups (15/sacrifice point) euthanized after 4 or 8 days after the start of treatment. See table 1.

Table 1: Study design

Group	Test substance	Treatment (ppm)	Animal numbers by scheduled kill time point				
			Day				
			2	4	8	15	29
1	Vehicle	0	1-15	16-30	31-45	46-60	61-75
2	Bicyclopyrone	5	76-90	91-105	106-120	121-135	136-150
3	Bicyclopyrone	500	151-165	166-180	181-195	196-210	211-225
4	Bicyclopyrone	5000	226-240	241-255	256-270	271-285	286-300
5	Phenobarbital Sodium Salt (PB)	1200	-	301-315	316-330	-	-

Table was taken from page 23 of the study report

Dose level selection: The dose levels were selected with the Sponsor after evaluation of the combined chronic toxicity and carcinogenicity study in rats (Charles River Study No. 458325). The dose levels were chosen to investigate the effect of the test substance on thyroid and liver parameters at the three dose levels of this study.

Diet preparation and analysis: Formulated diets were prepared, stored and used within the conditions established using methods developed under Charles River Study No. 423884, that demonstrated that dietary preparations of bicyclopyrone were stable for at least 15 days at ambient temperature. Bicyclopyrone doses were expressed as a dietary concentration (ppm) and were not adjusted for body weight or feed consumption. Controls were fed standard diet.

Diet preparations of positive control diets were made as necessary. In consultation with the Sponsor, 15 days stability when stored at ambient temperature was assumed. The preparation procedure involved preparing a 200 g premix, that is, a dietary concentration which was diluted by adding an appropriate amount of blank diet to produce the required final concentration of diet required.

Samples from diets were collected for analysis of concentration and homogeneity.

Concentration and homogeneity analysis results: Bicyclopyrone was not detected in the control diet. With one minor exception all bicyclopyrone-containing diets were considered to be accurately formulated with respect to both achieved concentration and homogeneity.

Observations: All animals were checked early morning and as late as possible each day for viability. Once each week, beginning during the week of the pre-trial period, each animal was removed from the cage and received a detailed clinical examination including appearance, movement and behaviour patterns, skin and hair condition, eyes and mucous membranes, respiration and excreta.

All animals were examined daily in their cage for reaction to treatment. The onset, intensity and duration of these signs were recorded.

Body weight: All animals were weighed twice during the pretrial period, and daily up to and including the day of necropsy.

Food consumption and test substance intake: The food consumed by each cage of animals was measured and recorded twice during the pretrial period and daily during the observation period.

The amount of test substance ingested was calculated for each cage (Groups 2-5) over every period of food consumption during treatment.

Water consumption: Water consumption was qualitatively monitored by visual inspection of the water bottles on a weekly basis.

Clinical pathology

Sample collection for thyroid function testing and tyrosine analysis: Blood samples (approximately 3.0 mL) were collected from the orbital sinus under isofluorane anaesthesia. The animals were not fasted prior to collection. Only the animals designated for euthanasia were sampled on the scheduled days (Table 2):

Table 2 Sample collection time points

Group no	Sample Collection Time Points				
	Day 2	Day 4	Day 8	Day 15	Day 29
1	x	x	x	x	x
2	x	x	x	x	x
3	x	x	x	x	x
4	x	x	x	x	x

Table taken from page 25 of the study report
X – sample collected

Blood samples were left at room temperature for approximately 1 hour before being centrifuged for approximately 15 min at approximately 3000 rpm (900 g). The resultant serum was separated into uniquely labelled plain polypropylene tubes. A total of 100 µL was separated for the tyrosine assay and the remainder was used for the thyroid function testing. The tubes were placed in a freezer set to maintain -20°C.

Thyroid hormone analysis: The serum samples were dispatched on dry ice to WIL Research Laboratories, LLC, 1407 George Road, Ashland OH 44805-8946 and were analysed for Tetraiodothyronine (Thyroxine, T_4), Triiodothyronine (T_3), and Thyroid Stimulating Hormone (TSH).

Terminal procedures*:* Animals were killed on their scheduled termination day (treatment days 2, 4, 8, 15 or 29) by exposure to a rising concentration of carbon dioxide and had their terminal body recorded followed by severance of major blood vessels for exsanguination.

Necropsy: All animals were subjected to a necropsy, which included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. Necropsy examinations consisted of an internal and external examination and recording of observations for all animals.

Organ weights: The thyroid gland and liver were weighed.

Microscopic examination: The thyroid gland and liver were processed to paraffin wax block from all animals, sectioned, mounted on glass slides, and stained with haematoxylin and eosin.

Histopathological evaluation of these tissues was undertaken for all animals.

Liver biochemistry: After weighing and sampling for histopathology, the remaining liver from all animals was collected, snap frozen and stored in a freezer set to maintain -80°C prior to despatch. Frozen samples from the designated animals detailed in the table below were despatched to Leatherhead Food Research (LFR), Randalls Road, Leatherhead, Surrey, UK for analysis of uridine diphosphate glucuronyltransferase (UDPGT) activity towards thyroxine as substrate.

Table 3: Liver biochemistry

Group	Animal numbers by scheduled kill time point (day)				
	2	4	8	15	29
1	1-6	16-21	31-45 a	46-51	61-66

2	76-81	91-96	106-111	121-126	136-141
3	151-156	166-171	181-186	196-201	211-216
4	226-231	241-246	256-261	271-276	286-291
5	-	-	316-330 b	-	-

Table was taken from page 26 of the study report

a Only animals 31-36 were analysed as part of this study.

b Group 5 animals were not analysed as part of this study

Each liver sample was labelled with the Charles River study number, animal identification, dose group and termination time point. The liver biochemistry was conducted under LFR Molecular Sciences reference number 5493/1.

Statistical analysis: The following statistical approaches were used on this study:
• All analyses were two-tailed for significance levels of 5% and 1%.
• If the variances were heterogenous, appropriate transformations were used in an attempt to stabilize the variances.
• Body weights, cumulative body weight gain, food consumption, absolute organ weights and thyroid data will be analysed initially by a one-way analysis of variance (ANOVA).
• Organ weights were analysed by analysis of covariance (ANCOVA) on final body weight.
• Summary values of organ to body weight ratios were not statistically analysed.
• Dunnett's test was used to compare the control and test substance group on continuous data parameters, regardless of whether the initial ANOVA or ANCOVA was statistically significant. For each thyroid endpoint, data from Groups 2-4 was compared to Group 1 and data from Group 5 compared to Group 1 separately.
• Micropathology incidence data was analysed using Fisher's Exact Test.

RESULTS AND DISCUSSION

Mortality: There were no unscheduled deaths during the observation period.

Clinical observations: There were no adverse clinical observations recorded in any animal receiving bicyclopyrone during the observation period that were considered to be related to treatment.

Signs observed such as bald areas, lesions, scabs or staining to fur, were considered to be typical findings observed at these laboratories in animals of this age and strain in this type of study and considered not to be related to treatment.

Piloerection, a staggering gait, irregular respiration and subdued behaviour were observed on Day 29 in one animal receiving 500 ppm bicyclopyrone. These were not observed in any other animal receiving 500 ppm or 5000 ppm bicyclopyrone, therefore, this isolated incident was considered not to be related to treatment.

Fast respiration, a rolling or staggering gait, reduced and increased activity and low body posture were observed up to Day 7 in animals receiving phenobarbital at 1200 ppm.

Body weight and cumulative body weight: Animals receiving 5000 ppm bicyclopyrone had a mean body weight loss on Days 1 and 2. These animals had a statistically significantly lower mean body weight and lower cumulative weight gain until day 14 of treatment, when compared with the controls.

Throughout the observation period, animals receiving 1200 ppm PB had a statistically significantly lower mean body weight and a statistically significantly lower mean cumulative gain, when compared with the controls.

Table 4: Intergroup comparison of mean body weights (g) (selected timepoints)

Group	Day				
	1	7	14	Overall change 0-14	Overall change 0-28
(control)	262.1 ± 16.0	288.0 ± 17.2	307.4 ± 20.2	44.5 ± 10.1	69.6 ± 16.1
5 ppm bicyclopyrone	262.3 ± 20.1	280.1 ± 21.2	295.9 ± 26.8	41.5 ± 13.4	69.5 ± 21.7
500 ppm bicyclopyrone	256.4 ± 18.4	277.7* ± 23.6 (↓4%)	300.6 ± 23.8	44.3 ± 10.1	69.7 ± 16.5
5000 ppm bicyclopyrone	248.3** ± 14.7 (↓5%)	269.4** ± 16.6 (↓6%)	290.0* ± 20.7 (↓6%)	30.6** ± 11.3 (↓31%)	54.7 ± 19.4 (↓21%)
1200 ppm PB	253.5 ± 16.3	268.5** ± 15.2	-	-	-

Data were taken from pages 45-48 of the study report

PB = Phenobarbital Sodium Salt

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Food consumption: Statistically significantly lower mean food consumption was observed on Day 1 in animals receiving 500 or 5000 ppm bicyclopyrone which continued to Day 7, when compared with the controls. On a few occasions after Day 7, mean food consumed for animals receiving 500 or 5000 ppm bicyclopyrone was lower when compared with the controls.

Food consumption was considered to be unaffected by treatment with 1200 ppm PB, although there was a statistically significant decrease from days 3-7 in this group.

Table 5: Intergroup comparison of mean food consumption (g/animal/day) (selected timepoints)

Group	Day				
	1	7	14	21	28
(control)	27.3 ± 2.5	26.8 ± 2.3	25.5 ± 2.8	26.5 ± 3.2	23.8 ± 1.3
5 ppm bicyclopyrone	26.8 ± 2.4	24.8 ± 1.9	26.6 ± 3.5	25.6 ± 2.7	24.6 ± 2.4
500 ppm bicyclopyrone	22.7** ± 2.0 (↓17%)	21.8** ± 2.3 (↓19%)	25.5 ± 3.8	25.3 ± 1.7	22.9 ± 2.4
5000 ppm bicyclopyrone	19.1** ± 4.4 (↓30%)	22.7** ± 3.1 (↓15%)	24.3 ± 4.9	24.2 ± 1.4	22.6 ± 5.1
1200 ppm PB	26.0 ± 3.0	21.6** ± 1.0 (↓19%)	-	-	-

Data were taken from pages 49-51

PB = Phenobarbital Sodium Salt

* Statistically significant difference from control group mean, $p<0.05$

** Statistically significant difference from control group mean, $p<0.01$

Table 6: Mean dose received (mg/kg/day)

Bicyclopyrone (ppm)	5	500	5000	1200 PB
Mean achieved dose	0.5	41.5	399.5	105.2

PB = Phenobarbital Sodium Salt
Data were taken from pages 35-44 of the study report

Water consumption: There were no differences in water consumption observed following visual inspection of the water bottles.

Thyroid Function:
T_3: Except for day 2 when the mean T3 levels were elevated, T3 levels showed no change on any other day at this concentration or animals receiving 1200 ppm phenobarbital.

Mean T_3 levels were lower following 4 (5000 ppm bicyclopyrone only), 8, 15 or 29 days of treatment in animals receiving 500 or 5000 ppm bicyclopyrone, when compared with the controls, achieving statistical significance at most time points.

T_4: Mean T_4 levels were lower following 4, 8, 15 and 29 days of treatment with 500 or 5000 ppm bicyclopyrone, when compared with the controls, achieving statistical significance at most time points. T_4 levels were statistically significantly lower in animals receiving 1200 ppm phenobarbital for 3 or 7 days, when compared with the controls.

TSH: TSH was not affected in animals receiving bicyclopyrone at any dose level tested, although there was a statistically significant decrease on day 2 in the 5000 ppm group. A statistically significantly higher TSH concentration was observed following 8 days of treatment with 1200 ppm phenobarbital, when compared with the controls.

Table 7: Summary of Total T_3 (ng/dL)

	Dietary concentration of bicyclopyrone (ppm)				PB (ppm)
Day	**0**	**5**	**500**	**5000**	**1200**
2	80 ± 12.5	106** ± 16.6 (↑33%)	92 ± 13.6	79 ± 13.5	-
4	85 ± 10.1	95 ± 12.0	80 ± 9.0	68** ± 15.3 (↓20%)	80 ± 16.8
8	84 ± 10.9	82 ± 17.3	67** ± 13.3 (↓20%)	74 ± 13.6	84 ± 16.9
15	85 ± 13.8	79 ± 9.0	74 ± 16.2	70** ± 8.9 (↓18%)	-
29	93 ± 17.4	86 ± 13.4	77* ± 14.0 (↓17%)	72** ± 11.3 (↓23%)	-

Data were taken from pages 253-254 of the study report
PB = Phenobarbital Sodium Salt
* Statistically significant difference from control group mean, $p<0.05$ (Dunnett's test)
** Statistically significant difference from control group mean, $p<0.01$ (Dunnett's test)

Table 8: Summary of Total T_4 (µg/dL)

Day	Dietary concentration of bicyclopyrone (ppm)				PB (ppm)
	0	**5**	**500**	**5000**	**1200**
2	4.9 ± 0.56	5.3 ± 0.63	4.8 ± 0.48	4.7 ± 0.59	-

4	5.0 ± 0.56	4.5* ± 0.45 (↓10%)	3.7** ± 0.38 (↓26%)	3.5** ± 0.37 (↓30%)	3.7** ± 0.43 (↓26%)
8	5.0 ± 0.51	4.7 ± 0.48	3.8** ± 0.43 (↓24%)	3.5** ± 0.44 (↓30%)	3.8** ± 0.54 (↓24%)
15	5.2 ± 0.68	4.4** ± 0.46 (↓15%)	3.8** ± 0.38 (↓27%)	3.6** ± 0.36 (↓31%)	-
29	5.4 ± 0.59	4.5** ± 0.64 (↓17%)	3.8** ± 0.40 (↓30%)	3.4** ± 0.39 (↓37%)	-

Data were taken from pages 255-256 of the study report
PB = Phenobarbital Sodium Salt
* Statistically significant difference from control group mean, $p<0.05$ (Dunnett's test)
** Statistically significant difference from control group mean, $p<0.01$ (Dunnett's test)

Table 9: Summary of Total TSH (ng/dL)

Day	Dietary concentration of bicyclopyrone (ppm)				PB (ppm)
	0	**5**	**500**	**5000**	**1200**
2	4.6 ± 0.98	5.0 ± 0.69	4.4 ± 0.72	3.5** ± 0.60 (↓31%)	-
4	4.8 ± 1.37	5.5 ± 2.72	5.9 ± 2.24	5.9 ± 1.02	5.4 ± 1.54
8	4.4 ± 0.85	4.9 ± 1.09	4.1 ± 1.31	4.9 ± 1.19	7.9** ± 2.17 (↑80%)
15	4.9 ± 0.92	5.1 ± 1.47	4.7 ± 1.36	5.2 ± 1.57	-
29	4.9 ± 1.21	5.1 ± 3.03	6.0 ± 2.68	5.6 ± 2.21	-

Data were taken from page 256 -257 the study report
PB = Phenobarbital Sodium Salt
* Statistically significant difference from control group mean, $p<0.05$ (Dunnett's test)
** Statistically significant difference from control group mean, $p<0.01$ (Dunnett's test)

Tyrosine analysis: Tyrosine serum levels were higher in all animals that received bicyclopyrone, when compared with the controls (data not listed in the study report). This was evident at every time point, with average increases to 6-, 14- and 15-fold higher than control mean for animals receiving 5, 500 or 5000 ppm bicyclopyrone, respectively. For animals treated at 5 ppm, tyrosine serum levels peaked at Day 4, declining to approximately half of the peak concentration on Day 29. For animals treated with 500 or 5000 ppm, tyrosine serum levels were similar at all time points in both groups, but substantially elevated compared to the 5 ppm dose group.

Terminal studies:
Necropsy findings: There were no necropsy findings that were considered to be related to treatment with bicyclopyrone or phenobarbital. The gross findings observed were considered incidental, of the nature commonly observed in this strain and age of rats, and/or were of similar incidence in control and treated animals.

Organ weights: Liver and thyroid weights were unaffected following 1, 3, 8 and 14 days of treatment with bicyclopyrone.

A statistically significantly higher mean covariant liver weight was observed on Day 29 for all doses of bicyclopyrone tested, when compared with the controls. These differences were also observed in the mean absolute liver weights, although not achieving statistical significance. Mean absolute and/or covariant liver weights were statistically significantly higher for animals receiving 1200 ppm PB for 3 and 7 days.

A statistically significantly higher covariant thyroid weight was observed on Day 8 in animals that received 1200 ppm PB, when compared with the controls.

Any other differences in organ weight, including those of statistical significance, were considered not to be related to treatment due to the lack of dose-response relationship and/or individual values were similar to those observed in the controls.

Histopathology: Liver and thyroid histopathology on day 29 are presented in table 10. Increased incidences of hepatocellular centrilobular hypertrophy were observed for animals that received 5000 ppm bicyclopyrone for 14 or 28 days. This was also observed for animals that received 1200 ppm PB for 3 or 7 days.

Statistically significantly increased incidences of thyroid follicular cell hypertrophy were noted in animals treated with 500 or 5000 ppm bicyclopyrone after 28 days of treatment. Increased incidences of thyroid follicular cell hypertrophy were also noted for animals that received 1200 ppm PB for 3 or 7 days.

Other microscopic findings observed were considered incidental of the nature commonly observed in this strain and age of rat, and were of similar incidence in control and treated animals.

Table 10: Summary of Microscopic Findings (Scheduled Euthanasia Day 29)

Day	Dietary concentration of bicyclopyrone (ppm)			
	0	**5**	**500**	**5000**
Liver (No. Examined)	15	15	15	15
Centrilobular hypertrophy	(0)	(0)	(0)	(10***)
Minimal	0	0	0	10***
Thyroid Gland (No. Examined)	15	15	15	15
Hypertrophy, follicular cell	(1)	(6)	(10**)	(7*)
Minimal	1	6	9**	6
Mild	0	0	1	1

Data was taken from page 501 of the study report
*Numbers in parentheses represent the number of animals with the finding,
Significantly different from the Control: *P <0.01, **P<0.01, ***P<0.001

Liver biochemistry: Liver protein activities are presented on tables 11 and 12. A statistically significant increase in hepatic microsomal protein content was noted for animals treated with 500 or 5000 ppm bicyclopyrone for 28 days.

Table 11: Intergroup comparison of hepatic microsomal protein content (mg/g liver)

Day	Dietary concentration of bicyclopyrone (ppm)			
	0	5	500	5000
1	27.5±1.84	27.8±3.94	29.3±3.66	29.0±1.11
3	29.9±3.36	30.7±2.48	27.4±1.58	28.2±2.77
7	29.2±1.30	29.3±2.85	28.7±1.51	28.7±2.16
14	25.5±2.53	27.8±5.31	24.4±2.19	25.5±2.25
28	26.9±2.50	29.3±3.89	31.7±1.03* (↑18%)	34.1±2.69** (↑28%)

Data were taken from page 520 -529 the study report

* Statistically significant difference from control group mean, p<0.05 (Dunnett's test)

** Statistically significant difference from control group mean, p<0.01 (Dunnett's test)

A statistically significant increase in hepatic microsomal UDP glucuronosyltransferase (UDPGT) activity (expressed as nmol/min/liver weight/kg body weight) was noted for animals treated with 500 or 5000 ppm bicyclopyrone for 28 days (see table 12). Statistically-significant differences were noted at other time-points in animals treated with 5000 ppm. Additionally, most other treatment groups and time-points were increased over the control group but without statistical significance.

Table 12: Intergroup comparison of hepatic microsomal UDP glucuronosyltransferase (UDPGT) activity towards thyroxine as substrate (nmol/min/liver weight/kg body weight)

Day	Dietary concentration of bicyclopyrone (ppm)			
	0	5	500	5000
1	21.32 ± 3.422 (100)	31.52 ± 10.850 (100)	32.25 ± 8.533* (151)	32.18 ± 3.549* (151)
3	25.60 ± 6.095 (100)	31.05 ± 8.668 (121)	27.56 ± 8.634 (108)	33.79 ± 4.841 (132)
7	27.03 ± 3.961 (100)	25.08 ± 7.601 (93)	35.68 ± 6.974 (132)	37.95 ± 5.215* (140)
14	22.04 ± 5.780 (100)	30.89 ± 13.937 (100)	25.32 ± 8.4569 (115)	25.59 ± 8.470 (116)
28	20.18 ± 4.861 (100)	30.06 ± 12.119 (100)	33.57 ± 5.950* (166)	38.13 ± 7.435** (189)

Data were taken from page 520-529 the study report

* Statistically significant difference from control group mean, p<0.05 (Dunnett's test)

** Statistically significant difference from control group mean, p<0.01 (Dunnett's test)

INVESTIGATOR'S CONCLUSIONS

This study has demonstrated that dietary treatment of male Han Wistar rats with bicyclopyrone results in increased tyrosine, decreased T_3 and T_4, increased thyroid follicular cell hypertrophy and increased liver weight associated with increased hepatocellular centrilobular hypertrophy and increased hepatic UDPGT activity. These data can be used as part of a weight-of-the-evidence approach to explain the mode of action for the increased incidence of thyroid follicular cell hyperplasia observed in male Han Wistar rats in a 2 year carcinogenicity study.

REVIEWER'S COMMENTS

In an in vivo Mode of Action study (MRID #47841990), the effect of bicyclopyrone (also known as NOA449280) on a number of parameters related to the liver and thyroid function was determined to elucidate the mode of action for thyroid hypertrophy/hyperplasia observed in a combined chronic toxicity and carcinogenicity study in rats. Groups of 75 male Han Wistar Crl:WI (Han) rats were fed diets containing 0, 5, 500 or 5000 ppm (0, 0.5, 41.5 and

399.5 mg/kg/day) bicyclopyrone (purity 94.5%) for a period of up to 28 days. A further group of 30 male Han Wistar rats were fed diets containing 1200 ppm phenobarbital (PB) for up to 8 days, which acted as a positive control with respect to thyroid function testing.

Treatment of male Han Wistar rats with 5, 500 or 5000 ppm bicyclopyrone resulted in dose and time-dependent increases in tyrosine, decreases in tetraiodothyronine (thyroxine; T_4), increases in liver weight and at 5000 ppm, increased incidences of hepatocellular centrilobular hypertrophy. In addition, treatment with 500 or 5000 ppm bicyclopyrone resulted in dose and/or time-dependent increases in hepatic UDGPT activity, decreases in triiodothyronine (T_3) and increased incidences of thyroid follicular cell hypertrophy. Mean body weight gain was lower throughout the treatment period for animals receiving 5000 ppm bicyclopyrone and lower mean food consumption was observed throughout the treatment period for animals receiving 500 or 5000 ppm bicyclopyrone.

Treatment of male Han Wistar rats with 1200 ppm PB resulted in time-dependent decreases in T_4, increases in TSH, increases in liver and thyroid weights, increased incidences of both hepatocellular centrilobular hypertrophy and thyroid follicular cell hypertrophy. This is consistent with previous findings with 1200 ppm PB (Charles River Study No. 459774), demonstrating its suitability as a positive control with respect to thyroid function testing.

This study has demonstrated that dietary treatment of male Han Wistar rats with bicyclopyrone results in increased tyrosine, decreased T_3 and T_4, increased thyroid follicular cell hypertrophy and increased liver weight associated with increased hepatocellular centrilobular hypertrophy and increased hepatic UDPGT activity. These data can be used as part of a weight-of-the-evidence approach to explain the mode of action for the increased incidence of thyroid follicular cell hyperplasia observed in male Han Wistar rats in a 2 year carcinogenicity study.

This study is classified as totally reliable (**acceptable/non-guideline)**. EPA, PMRA (Canada), and APVMA/OCS (Australia) agree on the regulatory decision and classification for this study.

REFERENCES:

Charles River Laboratories Study No. 458325. 104 Week Rat Dietary Carcinogenicity Study with Combined 52 Week Toxicity Study.

(Donald L, 2012)